MOLECULAR BIOLOGICAL STUDIES ON THE PARAMYOSIN GENE (*UNC-15*) MUTANTS AND INTRAGENIC REVERTANTS IN *CAENORHABDITIS ELEGANS*

KEIKO GENGYO-ANDO

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Molecular Biological Studies on the Paramyosin Gene(unc-15) Mutants and Intragenic Revertants in Caenorhabditis elegans

by

Keiko Gengyo-Ando

A dissertation for the degree of Doctor of Philosophy

Bioresources Science Division

Graduate School of Natural Science and Technology

Okayama University

January, 1991

Preface

The work described in this dissertation was carried out between April 1988 and December 1990 at Okayama University. The nematode Caenorhabditis elegans is now an indespensible organism for investigating muscle development. To understand molecular mechanism of thick filament assembly, mutants affecting paramyosin which is a major muscle component of thick filament of C. elegans, and its intragenic revertants, were analysed by molecular biological approaches.

With the exception of the technical events, this dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration. No part of this dissertation has been or is being submitted to any other university.

CONTENTS

Abbreviations

kb - kilobase pair(s)

MHC - myosin heavy chain

MLC - myosin light chain

SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PCR - polymerase chain reaction

FITC - fluorescein isothiocyanate

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1. Summary

Caenorhabditis elegans is a free-living, small soil nematode, and is a good model for investigating many problems of animal biology. Muscle development has been well studied in C. elegans, by using combination approaches of genetics, biochemistry, morphology, and molecular biology. Paramyosin forms a core of thick filament of C. elegans, and directly interacts with myosin heavy chains. It is encoded by a single gene, unc-15(I), and has almost α-helical coiled-coil structure(Kagawa et al., 1989). Mutants affecting paramyosin show uncoordinated movement phenotype resulting from abnormality of thick filament.

Objectives of this study are to know what types of sequence changes result in these paramyosin functional defects, and to define the functional domain of paramyosin molecule in the formation of thick filament. Understanding of how alterations of amino acid residues in paramyosin might affect the assembly processes, should provide crucial informations about the molecular mechanism of thick filament assembly.

This work is summarized as follows:

(1) Locarization of altered paramyosin within muscle cell

Strong semidominant allele, e73, was stained with polyclonal anti-C. elegans paramyosin antiserum following FITC(fluorescein-isothiocyanate)-conjugated second antibody. Compared with the regular pattern of A and I band observed in wild type Bristol N2,

e73 showed large aggregates in the end of body wall muscle cells. These structures probably correspond to paramyosin paracrystal which has been observed with electron microscopy(Waterston et al., 1977).

(2) Sequence analysis of unc-15 mutants and intragenic revertants

I sequenced the mutant unc-15 genes which were cloned or amplified from the genome. I determined the sequence changes of nine mutations including one nonsense, four missense, one deletion and three suppressor mutations. Eight of nine mutations are point mutations resulting in an amber codon or missense codons. C-terminal region of the paramyosin rod contains five mutations, suggesting this cluster may play an important role in in vivo thick filament assembly.

(3) Correlation of mutations to molecular assembly model of paramyosin

All mutation sites were located on the molecular model which is constructed by optimizing charge interactions between paramyosin rods. Remarkably, single charge reversals(e.g., glutamic acid to lysine) were found which either disrupted or restored filament assembly in vivo. The positions of the mutations within the paramyosin molecule support the models of paramyosin assembly and further suggest that the C-terminal region clustering five mutations and a site interacting with it play a key role in assembly.

(4) Immunological analysis of mutant paramyosins

Antigenisity of mutant paramyosin against three monoclonal

antibodies was investigated. Paramyosin produced in e1215 which had point mutation within the C-terminal cluster, had no cross-reactivity against monoclonal antibody NE1-6B2. The epitope of this antibody may exist in or near the C-terminal cluster. This result support our previous results which NE1-6B2 can recognize to the C-terminal region of the paramyosin rod. Further, amino acid sequence of this region has the characteristic property termed "weak spot", which may destabilize α-helical coiled-coil structure.

These sequence results and related immunological studies suggest that the C-terminal region is particularly important in both the structure and function of paramyosin molecules. Charge interactions between α-helical coiled-coil proteins have been postulated to determine the alingnment of many filamentous proteins, such as myosin heavy chain rod, paramyosin and α-keratin. My results show that single charge changes between the C-terminal region of the rod and its proposed interaction site in an assembly model can cause alterations in the in vivo paramyosin assembly properties, probably via charge interactions.

2. Introduction

2.1 Caenorhabditis elegans as an experimental organism

In 1974, Sydney Brenner reported "The Genetics of C. elegans", in which about 300 mutations were assigned to 100 genes. This paper showed that the free-living nematode Caenorhabditis elegans would promise to be an excellent organism for researching animal's behavior by a concerted genetic, ultrastructural, and behavioral investigation. During one or two decades, a lot of knowledge about the worm, that is anatomy, genetics, development and behavior, has been obtained, and C. elegans is now probably the most completely understood metazoan.

It feeds primarily on bacteria and reproduces with a life cycle of about 3 days under optimal conditions. It has two sexes, hermaphorodite and male. Most of the animal are hermaphrodites, which produce oocytes and sperm and can reproduce by self-fertilization. With a frequency of about 1 in 700 animals, males arise spontaneously which can fertilize hermaphrodites. Genetic informations are unidirectionally transferred from males to hermaphrodites, and thus genetic crosses can be carried out. The properties of short life cycle and self-fertilization of hermaphrodite is well suited for genetic analysis. About 700 genes have now been genetically identified. By seeking recessive lethal mutations and sterile mutations in a selected region of the genome and then assigning to complementation groups, the total number of essential genes has been roughly estimated 3000(Meneely & Herman, 1981; Rogalski et al., 1982; Rogalski &

Billie, 1985). C. elegans is a genetically simple organism, the haploid genome size is 1 x 10^8 nucleotide pairs, about eight times that of the yeast Saccharomyces or one-half that of the fluit fly Drosophila. 12 transcriptionally active genes were identified in 235 kb of cloned DNA(Heine & Blumenthal, 1986). If this spacing of 20 kb per gene were applicable to the entire genome, in theory, C. elegans would have about 5000 genes total(essential and unessential).

C. elegans is also anatomically simple, adult hermaphrodite has only 959 somatic cells. Because of the tranparency of its body, cell division pattern can be easily observed by differential interference contrast microscopy(Nomarski). The entire cell lineage from fertilized egg to adult has been completely described(Sulston & Horvitz, 1977; Sulston et al, 1983). In addition to the knowledge of the locations and characteristics of all somatic cells in the adult hermaphrodite and male, the pattern of synaptic connectivity within the nervous system has been gained from anatomical work(White et al., 1986). Further, neurophysiological function has been studied by neurotransmitter analysis(Sulston et al., 1975; Lewis et al., 1980, 1987) and electrophysiological analysis of the homologous nervous system in the large nematode Ascaris suum(Stretton et al., 1978, 1985; Walrond et al., 1985).

Various mutants have been isolated, and genetic approaches of them have revealed many development processes, such as vulva formation, sperm maturation, sex determination, muscle assembly, neuronal differentiation and dauer larva formation. By tranposon-tagging method being recently introduced to cloning of genes

mutationally defined, all of the mutant genes can be cloned, and investigated structure and function of them with molecular analysis. Cloned genes can be also microinjected into the worm, thus their functional expression *in vivo* should be examined(Fire, 1986; Fire & Waterston, 1989). Physical mapping of the entire genome has been almost completed(Coulson *et al.*, 1986, 1988). As correlating the physical and genetic map progresses, ultimately, separate gene isolation projects will become unnecessary. With these experimental attributes and the wealth of descriptive information, *C. elegans* should be useful model for investigation of various aspects in animal biology.

2.2 The problems in muscle biology

Studies of muscle is mainly divided into two parts, the assembly of the myofilament lattice during development and the contractile process itself. As muscle has a well-organized structure with many components, the investigation of its proper assembly should reveal general principles applicable to the spatial differentiation of the organism. There are two unsolved problems in muscle development---that is how each muscle proteins are expressed with time- and tissue-specific pattern, and how these do assemble in the muscle cells. Key proteins regulating myogenesis, MyoD(Davis *et al.*, 1987), myogenin(Wright *et al.*, 1989) and myf-5(Braun *et al.*, 1989), have been identified, but other important regulatory factors which define the variety of muscle forms---smooth muscle, cardiac muscle and striated muscle,

are still unknown. Nevertheless the propensity of major muscle components for self-assembly have been examined *in vitro*, the assembly process that these components yield the above muscle forms *in vivo* are only poor understood. During contraction, it is known that myosin-containing thick filaments slide past actin-containing thin filaments with hydrolysis of ATP by a myosin-linked ATPase. However, there has little knowledge about mechanism which chemical energy of ATP is transduced to the mechanical energy of movement.

2.3 Muscle of *C. elegans*

Molecular mechanisms of muscle structure and function have been mainly studied in *C. elegans* and *Drosophila*(Deak *et al.*, 1982; Karlik *et al.*, 1984; Mogami & Hotta., 1981; Mogami *et al.*, 1986). Through combined genetic, biochemical, morphological and molecular studies, the genes for the major muscle components have been identified(Brenner, 1974; Waterston *et al.*, 1980; Zengel & Epstein, 1980; Waterston, 1988). This combination of approaches has been very powerful to understand analogous processes in procaryotes, such as bacteriophage morphogenesis (Wood & King, 1979), and is proving successful in *C. elegans*.

C. elegans has 135 muscle cells of the approximately 1000 cells in the adult hermaphrodite. These include: 95 body-wall muscle cells, responsible for movement of the animal; 20 pharyngeal muscle cells, responsible for pumping food into the intestine; 16 vulval and uterine muscle cells, responsible for expel eggs; 2 intestinal muscles; 2 anal muscles. The body-wall

musculture is most prominent, in both cell number and total mass. It is arranged in four stripes, lying left and right subdorsally and left and right subventrally. Each muscle cell is spindle-shaped, and contains myofilament lattice which forms obliquely striated array. This structural unit is analogous to the sarcomere of vertebrate striated muscle. The second-most abundant muscle, pharyngeal musculture, is constituted from muscle filaments which are organized as single sarcomeres. The other muscles involving pharyngeal muscle, have not been studied as much as body wall muscle, but providing the interesting examples of differential gene expression within multigene families.

The structures of muscles of C. elegans is very similar to that of vertebrate muscles, but there are differences between them in three aspects. First, as mentioned above, nematode muscle is obliquely striated. Second, the thick and thin filaments differ in size and composition from those of vertebrates. The thick filaments in C. elegans are 10 μm in length, and longer than in vertebrates(1.6 μm). The thin filaments in C. elegans are also longer(6 μm) than in vertebrates(1 μm). The core protein, paramyosin, is contained in nematode thick filament like those of other invertebrates, while the thick filaments of vertebrates are only composed of myosin. Third, C. elegans muscle differ from vertebrate muscle in its attachment. The attachment plaques are found in half-I-bands at the myotendinous junctions of skeletal muscle and the intercalated disks of cardiac muscle. All tensions produced by sliding of the thin filament are transmitted between cells via attachment plaques. In C. elegans, the attachment plaques analogous to those of vertebrates also exist in the ends

of the muscle cells and some tension is transmitted between cells via these structures. However most seems to be transferred directly to the cuticle through a series of lateral attachments. Both the thick filaments and the thin filaments attach to the dense bodies(Z lines) and M lines respectively, which are firmly anchored in the basement membrane(Francis & Waterston, 1985). The basement membrane is attached through the hypodermis to the cuticle by a regular array of half-desmosomes and attached filaments. This series of attachments cause the cuticle to bend in contraction. Like other nematodes, acetylcholine(ACh) and ACh agonists(Nicotine, Levamisole, etc) can cause contraction of the body wall muscle cells in C. elegans(Sulston et al., 1975; Lewis et al., 1980). The processes electrical stimulation causing a muscle cell to contract, termed excitation-contraction (E-C) coupling are still unclear. It has been suggested contraction of skeletal muscle is triggered by the release of calcium ions from the sarcoplasmic reticulum (SR) following depolarization of transverse tubules (T-tubules)(Tanabe et al., 1988). Nematode muscle contains the SR, however, no equivalent to the T-tubule system does not exist. The transmission of electrical signals triggered the SR Ca^{2+} release in neuromuscular junctions should be fundamentarily similar to that of vertebrates, but such differences in the structure of muscles may reflect the specific signaling systems in C. elegans.

Mutants affecting the body wall muscles are less motile, but visible. Many muscle defective mutants have been isolated with the uncoordinated movement phenotype(unc), and more than 25 genes

involving the muscle formation and function of the body wall muscle have been identified. Polarized light microscopy can be used to detect abnormalities of muscle structure in the living animal so that mutants affecting this structure can be readily distinguished from other mutants affecting movement. Further detailed observation can be undertaken with electron microscope. Cloning and sequencing of such mutant genes have revealed the molecular properties of many muscle components. Alternatively, some genes have been obtained with molecular approach by using the homologous genes which had been characterized in other organism.

The thick filaments of the obliquely striated body wall muscle are composed mainly of structural components, myosin and paramyosin, in a 1:1.5 molar ratio. Myosin is composed of six subunits, two identical myosin heavy chains(MHC) and two pairs of myosin light chains(MLC). Almost half the MHC is the α -helical coiled-coil rod and the remainder is the globular heads which possesses actin-binding and ATPase activities. In C. elegans, MHC has been studied intensively by genetic, biochemical, and molecular biological approaches(Waterston, 1988). Two different MHCs, termed MHC A, and MHC B, are present in each cell, with the MHC A isoform restricted to the central portions and the MHC B isoform localized in the terminal portions of each thick filament(Miller et al., 1983, 1986). In pharynx, other two MHC isoforms, MHC C and MHC D, are expressed. MHC B is the most abundant, comprising about 70 % of the total myosin heavy chain in the adult, MHC A accounts for about 20 %, and MHC C and D accounts for 5 % each. MHC A, B, C and D are encoded by the

genes, myo-3, unc-54, myo-2 and myo-1, respectively. Genetic analysis of many myosin mutants, mainly unc-54 defective, have been undertaken(Epstein et al, 1974). Complete sequence of four MHC genes have been reported(Karn et al., 1983a; Dibb et al., 1989) Paramyosin, a core protein of the thick filament in many invertebrates(Cohen et al., 1970, 1971; Levine et al., 1976), is encoded in C. elegans by a single gene, unc-15(Waterston et al., 1974, 1977; Rose & Baillie, 1980), and directly interacts with both myosin isoforms A and B(Miller et al., 1983, 1986; Epstein et al., 1985, 1986) in body wall muscle cells. Kagawa et al(1989) cloned the paramyosin gene, unc-15, by screening genomic expression library of C. elegans with specific antibody against paramyosin, and sequenced it completely. The unc-15 gene is composed of ten exons encoding 866 amino acids. Amino acid sequence promised that paramyosin is a typical α -helical coiled-coil protein, since throughout most of its length showed the expected heptad repeat of hydrophobic amino acid residues. Short non-helical segments are however present at both the N- and C-termini, with the former likely to contain a phosphorylation site(Schreifer & Waterston, 1989). A common structural motif is the filamentous components of striated muscle is the α -helical coiled-coil, which is found also in intermediate filament proteins. The coiled-coil is formed from the side to side association of monomers along a hydrophobic face of the α -helix. In turn, charged residues are restricted to the outer surface of the coiled-coil and are thought to be important in dictating interactions between dimers in the formation of filaments. The

interactions of α-helices is important as well in many other proteins of biological interest such as the transmembrane domain of receptor molecules(Noda et al., 1984), the leucine zipper motif of nuclear transforming proteins and eucaryotic transcriptional regulatory proteins(Landschulz et al., 1988; O'sea et al., 1989). Nematode paramyosin has about 40% identity with myosin heavy chain rods and shows a similar 28-residue repeat of charged residues(Kagawa et al., 1989). These charged residues are thought to be responsible for assembly of myosin and paramyosin into filaments. The relatively simple structure of these α-helical coiled-coil proteins has facilitated the construction of molecular models for myosin and paramyosin, despite the absence of crystal structure(McLachlan & Karn, 1982; Kagawa et al., 1989). Charge-mediated interactions between paramyosin rods, between myosin rods and between paramyosin and MHC B rods have been calculated for different alignments of the molecules and models of the most stable arrangements have been constructed which are consistent with known features of thick filament structure.

Genetic analysis suggests that unc-22 product interacts with MHC B. unc-22 mutants show the "twitching" phenotype, and have disorganized muscle, although the degree of abnormalities is subtle(Moerman & Baillie, 1979; Waterston et al., 1980). Twitching occurs within the subcellular region of each muscle cells, seems to be spasmodic. These facts suggest that unc-22 product involves in regulation of the contractile cycle. The unc-22 gene has been cloned by transposon-tagging(Moerman et al., 1986). Molecular analysis shows that the unc-22 gene encodes over

600 kilodaltons proteins, termed "twitchin", which contains homologous region to the kinase domain of chicken smooth muscle myosin light chain kinase(csmMLCK). The remainder of the unc-22 protein is composed of repeated blocks of two different 100 amino acid motifs, copies of which are also present in csmMLCK. Twitchin may regulate contraction by phosphorylating the regulatory MLCs.

The thin filaments contain actin, tropomyosin, and presumably troponin. Actin, a globular protein, is extremely conserved in structure throughout the animal and plantkingdoms. In muscle system, actin binds to and activate myosin resulting in actomyosin-based motility. Actin gene family has been cloned and completely sequenced(Files et al., 1983; Krause et al, 1989). C. elegans has four actin genes(act-1, act-2, act-3, and act-4) in the genome, each of which expresses a unique mRNA. The cloning and sequencing of the tropomyosin gene is in progress(Harris & Epstein, 1977; Kagawa unpublished).

In addition to thick and thin filaments, muscle contains the accessory proteins such as M-lines, dense bodies, and intermediate filaments. More than 40 antibodies were prepared against these accessory proteins and used to not only establish the accessory structures but also recognize new structures not identified previously. The structure of vinculin, the basal component of the dense body, has been revealed by molecular analysis with one of these antibodies(Barstead & Waterston, 1989).

2.4 Paramyosin mutants

Paramyosin has been first discovered in the retractor muscles of bivalves of phylum Mollusca. The structure of paramyosin molecule of clam Mercenaria is most α-helical coiled-coil has been documented by X-ray diffraction studies(Bear and Selby, 1956; Cohen & Szent-Gyorgyi, 1957). It is suggested that paramyosin might involve in "catch" mechanism of bivalve retractor muscles, but its roles is still unclear. Ikemoto and Kawaguchi(1967) reported that it could regulate the length of thick filament. It can form the paracrystalline array resulting from self-assembly at appropriate conditions.

In C. elegans, several unc-15 mutants having highly disorganized muscle structure resulting from functional defects of paramyosin have been isolated(Brenner, 1974; Waterston et al., 1977). The number of unc-15 alleles is fewer than that of unc-54 alleles. These include both mutants which lack paramyosin entirely and likely missense alleles in which paramyosin accumulates at normal levels. In the absence of paramyosin, MHCs still aggregate, but the structures formed bear little resemblance to normal thick filaments and do not support contraction. The putative missense alleles typically show large birefringent aggregates, thought to consist primarily of paramyosin. Both extragenic suppressors and intragenic revertants have been obtained for a semi-dominant missense allele, e73(Riddle & Brenner, 1978; Brown & Riddle, 1985).

In this study, by locating the mutations of these unc-15

mutants and intragenic unc-15 revertants within the paramyosin molecule I hoped to identify regions critical for assembly. Further, with the molecular models of paramyosin-paramyosin interactions, a more detailed understanding of how these mutations might affect assembly might emerge.

3. Materials and Methods

3.1 Worm strains and culture

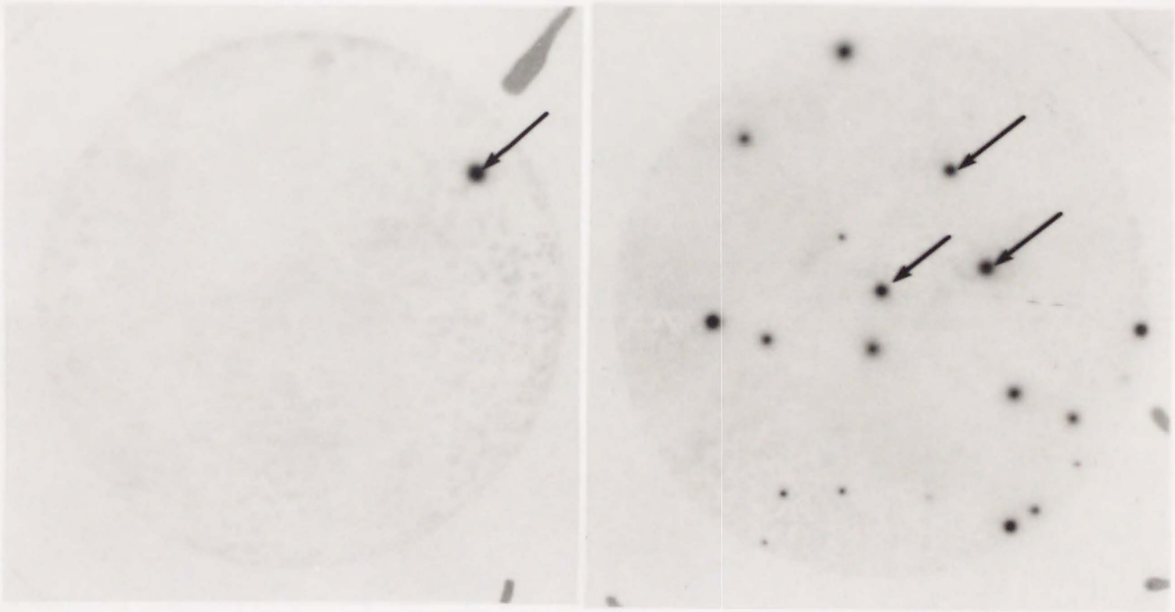
The wild-type parent of all strains in this study is *C. elegans* var. Bristol strain N2(Brenner, 1974). *unc-15*(e73), *unc-15*(e1214), *unc-15*(e1215), *unc-15*(e1402)(Waterston *et al.*, 1977) and *unc-15*(e73m193), *unc-15*(e73m208), *unc-15*(e73m209)(Brown & Riddle, 1985) were obtained from the Caenorhabditis Genetics Center. *unc-15*(su228) and *unc-15*(su2000) were obtained from Dr. Ann Rose(University of British Columbia, CANADA). Worm culture and handling followed conventional methods(Sulston & Hodgkin, 1988).

3.2 Cloning *unc-15* gene

Genomic DNA was prepared from each strain as previously described(Wills *et al.*, 1983). General methods for manipulating DNA are described in Maniatis *et al.*(1982). DNA was completely digested with BamHI, and 10 kb fragments containing the entire *unc-15* gene(Figure 8(1)) were purified with Gene Clean^{TM}(BIO 101). These fragments were ligated into purified arms of the lambdoid 2001 phage vector(Karn *et al.*, 1983b). After *in vitro* packaging of ligated DNA, the *E. coli* selective strain Q359 was transfected. Ten to fifty positive clones were isolated by screening $5X10^4$ plaques with ^{32}P labeled DNA probes(Figure 1).

Figure 1: The plaque hybridization of unc-15 mutant genome library.

Autoradiographs of plaque hybridization of e1214 λ 2001 genome phage library are shown. Arrows indicate the positive clones hybridized with the wild type unc-15 gene fragments.



3.3 DNA sequence analysis

Inserted DNAs of lambda genomic clones were purified, and the HindIII 5 kb fragment(Figure 8(3)) and the BamHI-EcoRI 2 kb fragment(Figure 8(2)), were subcloned into M13 phage vectors(Sanger et al., 1977), and sequenced by using Sequenase^{TM}(US Biochemicals). I completely sequenced the unc-15 coding regions of three mutants and three revertants with 15 synthetic oligonucleotide primers(Figure 8 and Table 1).

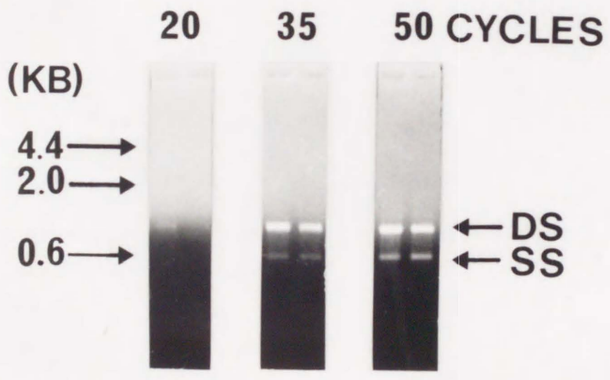
3.4 Asymmetric PCR methods

After 0.5-1 μg of C. elegans genomic DNA was completely denatured at 100°C for 10 min, 20 μM of each dNTP and 2.5 units of Taq DNA polymerase(PERKIN ELMER CETUS) and 10 X reaction buffer[500mM KCl/100mM Tris-HCl, pH8.3/15mM $MgCl_2$ /0.1%(w/v) gelatin] were added for a 100 μl reaction. Primers were set to 100 pmol:1 pmol, and then samples were overlaid with mineral oil to prevent evaporation. 50 cycles of PCR could yield 2-3 pmol of single stranded DNA which was processed for direct sequencing(Figure 2; Gyllensten & Erlich, 1988).

3.5 Direct sequencing

Ten pmol of primers were phosphorylated with 1 μl of γ-^{32}P-dATP(7,000 Ci/mmol, 185mCi/ml, ICN) and 1 unit of T4 polynucleotide kinase by using MEGALABEL(Takara Shuzo). 2 pmol of labeled primers were annealed to the 0.5 pmol of amplified single stranded DNA fragments in 5 X sequencing buffer*. 3 units(2 μl)

Figure 2: Amplified DNA fragments with PCR method.
1059 bp(nt) of DNA framents are amplified by using primers KG6 and KG16, and are electrophoresed on 0.75% agarose gel. After 30 cycles of amplification, the rate of production of double stranded DNA(DS) declines, while the single-stranded(SS) product accumulates only at a linear rate until 50 cycles. The single-stranded DNA produced by 50 cycles of amplifing reaction, were used to direct DNA sequencing.


20
35
50 CYCLES
(KB)
4.4
2.0
0.6
DS
SS

of T7 DNA polymerase*, 2 μl of 0.1M DTT* and 2 μl of distilled water were added to the reaction(10 μl). The reaction(17 μl) was divided into four wells of a microtiter plate containing 2.5 μl of cold NTP mix*, and incubated for 5 min at 42°C. Samples were incubated for 2 min at 80°C in 4 μl of stop solutions* and electrophoresed on buffer gradient acrylamide gels at 2000 V for 3hrs. Gels were processed by standard protocol. Reagents marked with an asterisk are from Sequenase™(US Biochemicals).

3.5 Immunological procedure

SDS-PAGE analysis and immunological methods were performed as described by Kagawa et al.(1989). The horse radish peroxidase-conjugated rabbit anti-mouse immunoglobulin G(P260, DAKO) was used in conjunction with monoclonal anti-C. elegans paramyosin antibodies.

3.6 Immunofluorescence and microscopy

Immunofluorescence was performed according to the method of Priess & Thomson(1987). The fixed animals were stained with anti-paramyosin polyclonal antibody R224 overnight at 4°C, then rinsed in three changes of PBS. The slides were further stained with the fluorescein-isothiocyanate-conjugated swine anti-rabbit immunogloblin G(F205, DAKO), rinsed, and then mounted in retardant buffer containing diazabicyclooctane. Immuno-fluorescence microscopy and polarized light microscopy were done using a Zeiss microscope(Axioscope) equipped with Plan-Neofluar lens(40x, 100x).

4. Results

4.1 Muscle structure of unc-15 mutants

The unc-15 alleles studied here can be divided into three phenotypic classes. The first class consists of the null allele, e1214, which results in complete paralysis of the adult. The second class consists of alleles producing a defective paramyosin, e73, e1215, e1402, su228 and su2000. These mutants vary in the severity of the mutant phenotype(Table 2) and accumulate normal levels of paramyosin of a size indistinguishable from that of the wild type strain N2(Figure 14A; Waterston et al., 1977). The third class consists of three pseudorevertants of the semidominant allele e73. These have motilities greater than e73 but less than that of N2, in the order N2 > e73m208 > e73m209 > e73m193 > e73(Brown & Riddle, 1985). Figure 3 shows the wild type animal with normal movement, and a typical unc-15 mutant, e73, which is included in the second class on culture plates.

C. elegans has obliquely striated array of myofilament lattice in the body wall muscle cell which can be directly observed with a polarized light microscopy(Figure 4A). The second major musculature, pharyngeal muscle, contain the myofilament consisting of single sarcomere(Figure 4B). Instead of the regular A and I bands, e73 has birefringent needle-shaped aggregates, which have been postulated to represent paramyosin paracrystals(Figure 5). Each of the mutants of the second class have also these abnormal structures(Waterston et al., 1977; data

Figure 3: Phenotypes of the wild type Bristol N2 and a typical unc-15 paramyosin mutant e73 of C. elegans. Adults and larvae of wild type(A), and e73(B) on culture plates. All animals are hermaphrodites. The wild type desplays a smooth sinuous movement. e73 move more slowly than the wild type, and the adults of which are often accumulating in "rafts". Bars represent 1 mm.

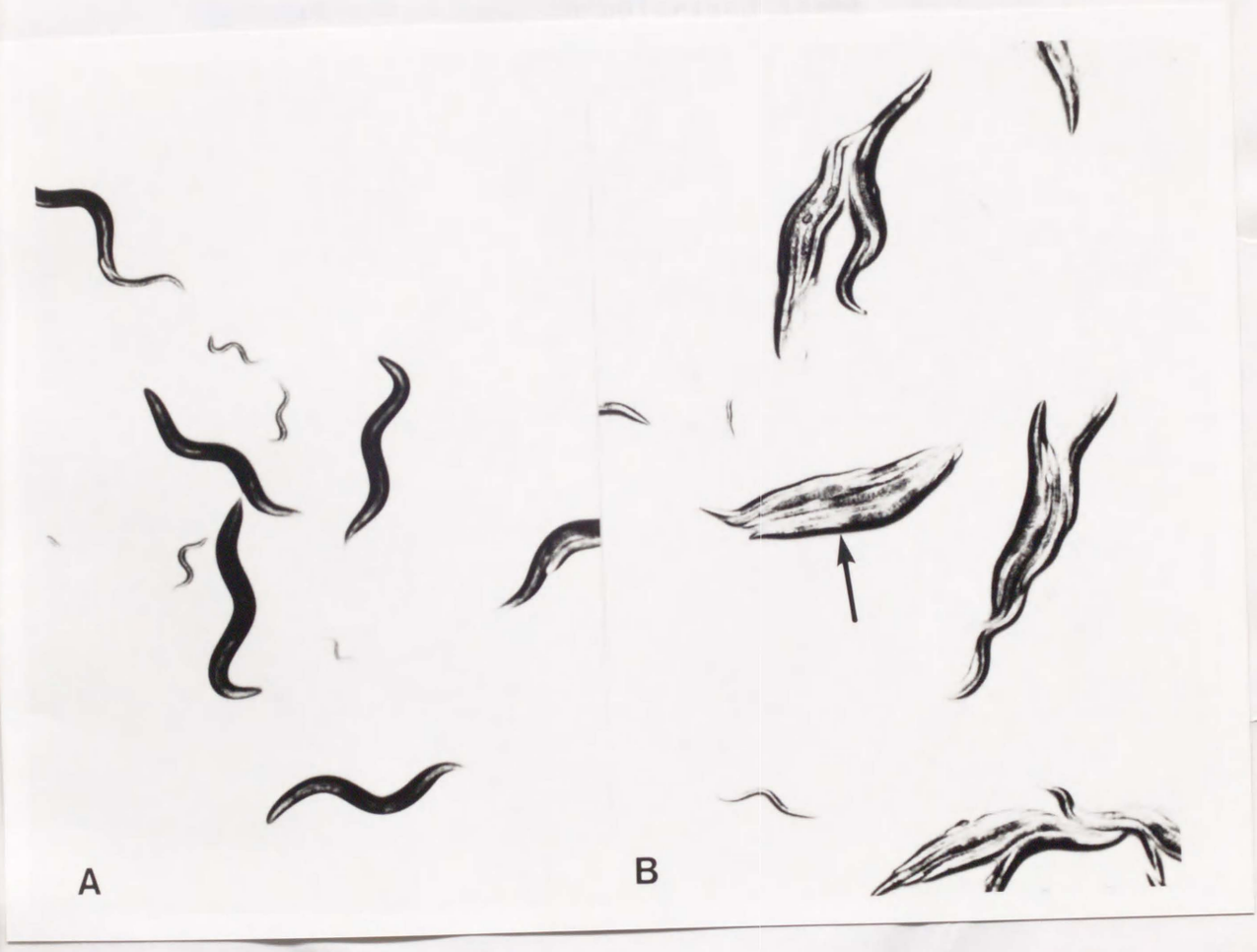
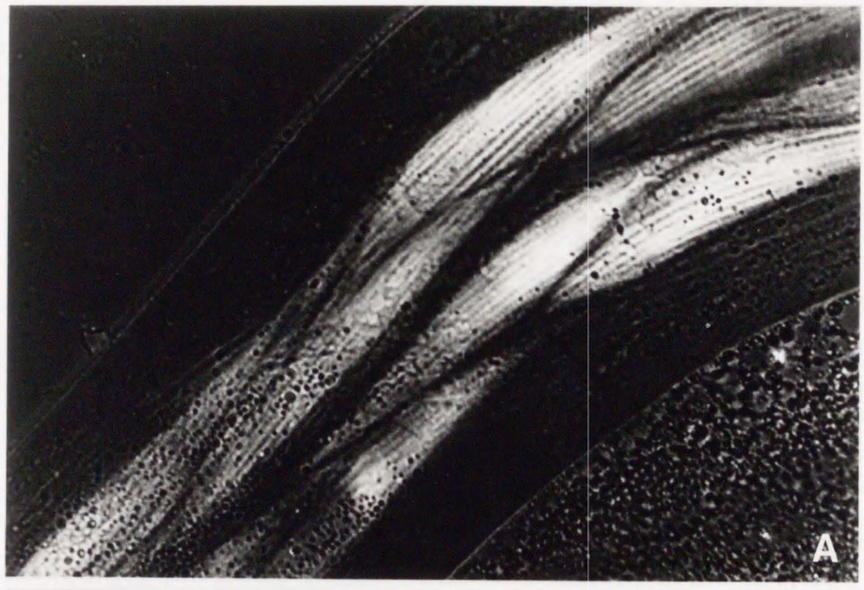

A
B

Figure 4: Birefringence of nematode body musculature of adult hermaphrodite seen in polarized light.
(A); The body wall musculature viewed from dorsal or ventral aspect. The bright A bands containing thick filaments, and the dark I bands containing thin filaments, alternately array in the spindle-shaped body wall muscle cells. (B); The pharyngeal musculature. Pharynx is divided into four regions; the anterior procorpus, a bulb-shaped metacorpus, a cylindrical isthmus, and a terminal bulb. Arrow indicates a terminal bulb with radially oriented muscle filaments. Bars represent 25 μm.


A


B

Figure 5: The differences of body wall musculature in wild type and e73.

The structure of the body wall muscles of wild type N2 (A) and e73(B), are viewed from lateral aspect. Unlike the regular pattern of A and I bands observed in (A), e73 shows needle-shaped structures(arrow) which probably correspond to paramyosin paracrystals. Bar represents 50 μm.

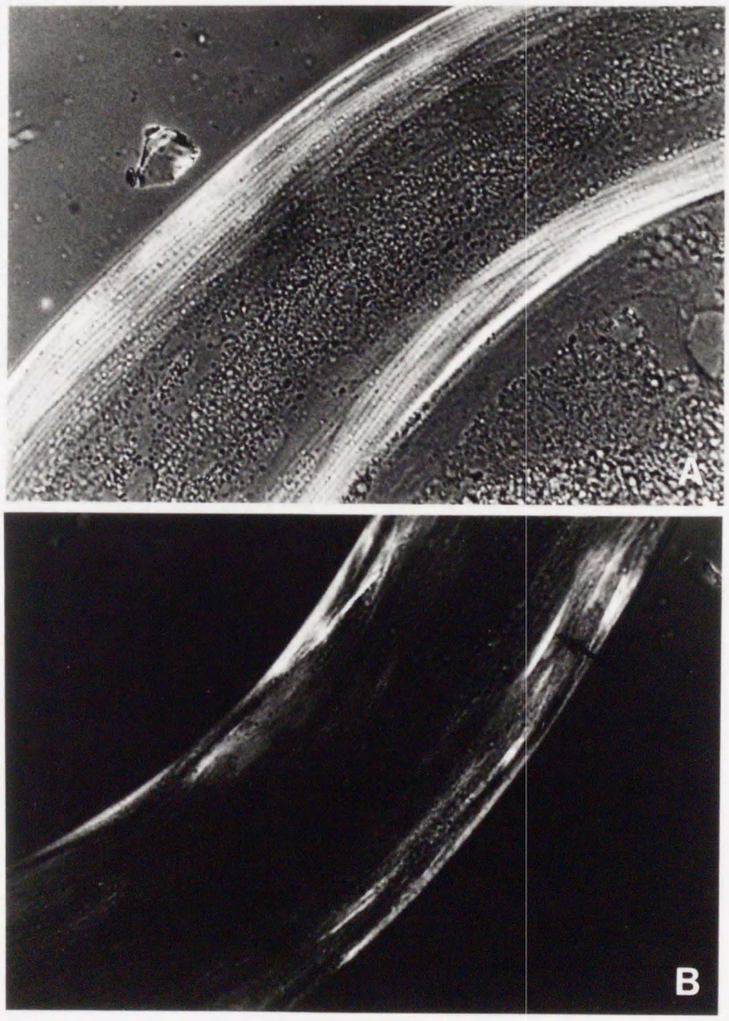
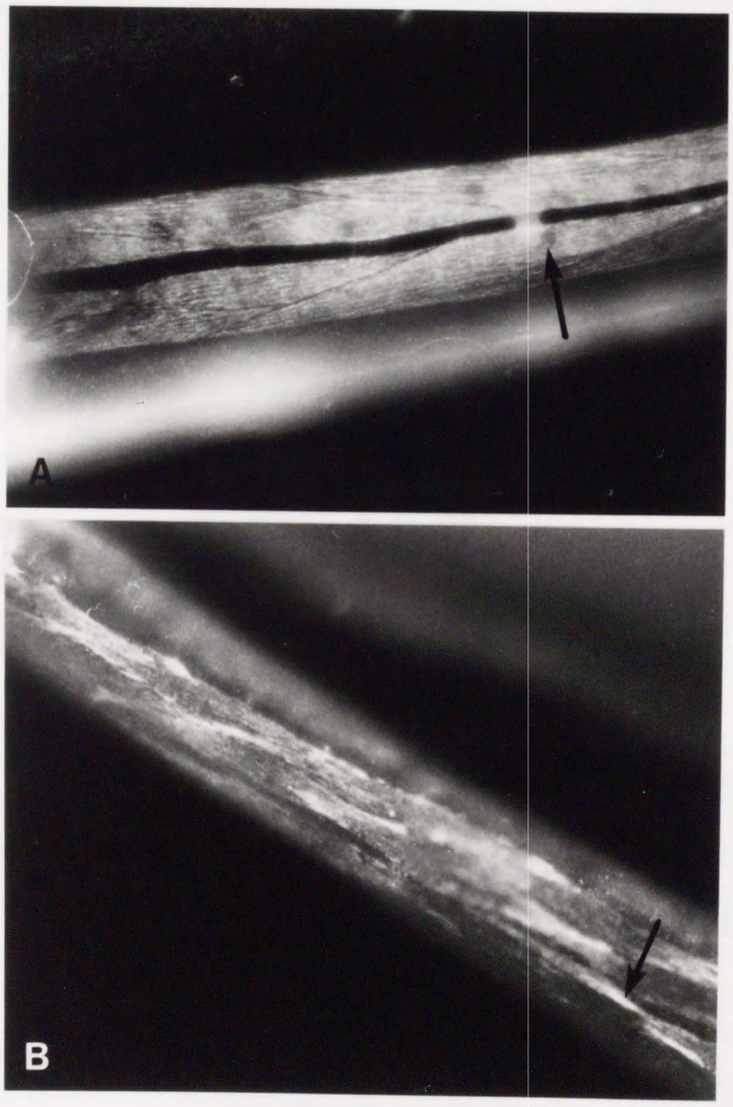

A
B

Figure 6: Immunofluorescent staining of body wall musculature. (A); Adult hermaphrodite of wild type N2 stained with polyclonal anti-paramyosin antibody R224. Arrow points to mononucleus in body wall muscle cell. (B); Adult hermaphrodite of e73 stained with R224. The large aggregates are shown at the end of the muscle cell. (C); The body wall muscle of e73 observed in higher magnification with confocal lens. Bars represent 50 μm for (A) and (B), 10 μm for (C), respectively.


A
B

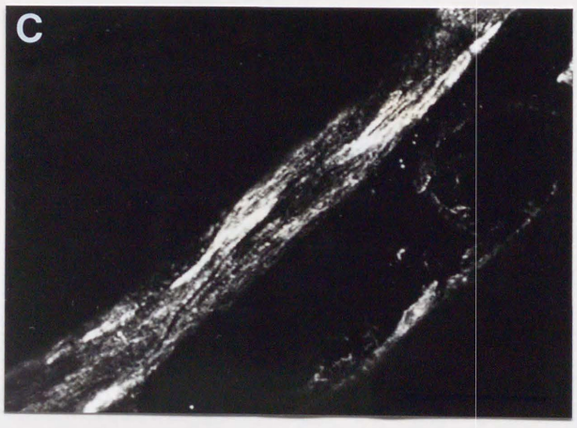
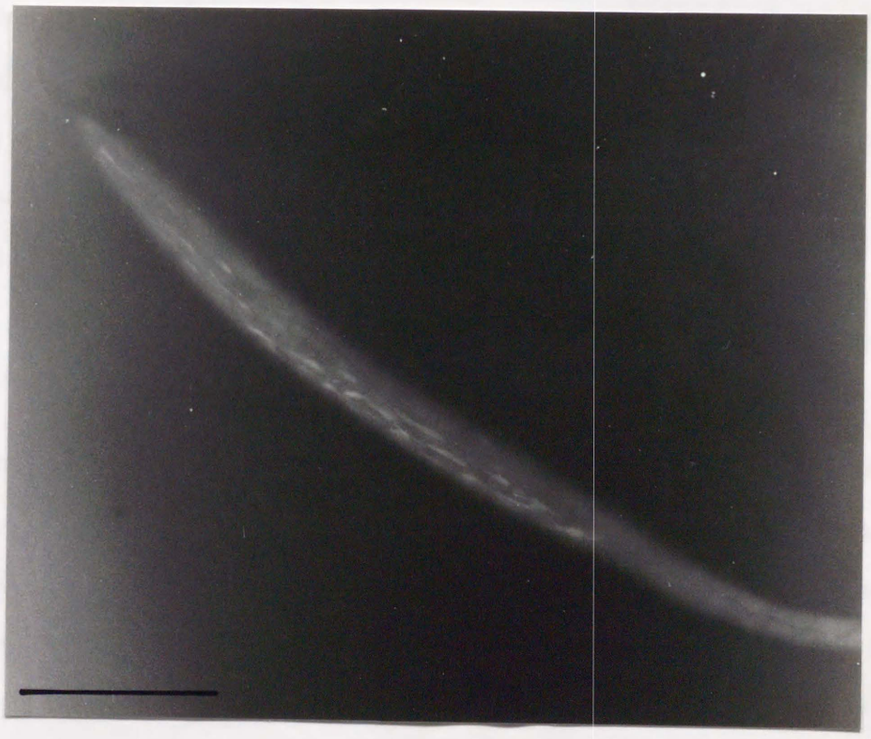

C

Figure 7: Immunofluorescent staining of e73 larva.

e73 larva(L3) stained with polyclonal anti-paramyosin antibody R224. Paramyosin aggregates are seen throughout in the body.

Bar represents 10 μm.



not shown). The pseudorevertants also have similar structures but these are generally smaller and less numerous than those found in e73. To directly determine the distribution of altered paramyosin in the muscle cells, we stained e73 animals, which have severely impaired motility, with anti-paramyosin antibody, visualized with indirect immunofluorescense(Figure 6 and 7). Instead of the regular pattern of distribution observed in the wild type strain N2(Figure 6A), adult hermaphrodites of e73 animals showed needle-shaped structures similar in size, number and distribution to the birefringent structures visible in polarized light microscopy(Figure 5B and 6B). The similar aggregates were also seen in larva(but not seen in L1 and L2 because of their small size)(Figure 7).

4.2 Molecular identification of mutations

To understand how and why these abnormalities in paramyosin disrupt thick filament assembly, I determined the sequence of the unc-15 gene of each mutant. I cloned the entire unc-15 gene from the strains e1214, e73 and e1402, and completely sequenced the exons(Figure 8; See Materials and Methods) by using 15 synthetic oligonucleotides as primers(Table 1). Figure 9 shows the DNA sequence ladders of the mutation sites on sequencing gels. The mutations identified by these sequence analysis were locarized in the complete DNA sequence of wild type unc-15 gene(Figure 10). Each of these alleles had a single point mutation in the unc-15 gene. The unc-15(e1215) gene was also cloned but sequence analysis was focused on the C-terminal region containing the

Table 1: Synthetic oligonucleotide primers.

Synthetic oligonucleotide primers used in sequence analysis and amplification of the mutant unc-15 genes are indicated.

(A) Antisense strand

Name	Sequence	Position
HK6	TTGGGGTTTTGGCAGTG	5517-5533
HK7	ATTATACACATGATACT	8392-8408
HK8	TGCGTCAGAGGAAGAAA	8838-8855
KG16	CGTTTAACATAAAATCT	10901-10917

(B) Sense strand

Name	Sequence	Position
KG1	ACGAACTTGCCAACAAG	8281-8297
KG2	TTTCTGGACATTAAAAA	8856-8872
KG3	CCGCAAGAAGATGCTCC	9107-9123
KG4	GAACTCCGTGCCGTCAA	9357-9371
KG5	ACCGCGCTCAACGTGCC	9607-9623
KG6	TTTTCAACATTCAACCC	9858-9874
KG7	GACCGCTCTCGACAACG	10108-10124
KG8	CAAGAACACTCGATGAA	10358-10374
KG9	AGAGCACAAGAACTTCG	10609-10625
KG10	TCTTGAAACAATTAATT	6558-6574
KG11	TTTTTATGAATTTCAAT	6817-6833

Figure 8: The physical map of the unc-15 region.

Ten exons are drawn as shaded boxes, introns as gaps. Restriction enzyme site abbreviations are: B, BamHI; H, HindIII. (1) BamHI 10 kb fragment cloned into the lambdoid 2001 phage vector. (2) BamHI-EcoRI 2 kb fragment cloned into an M13 phage vector. (3) HindIII 5 kb fragment cloned into an M13 phage vector. (4) Antisense single stranded DNA fragments emzymatically amplified by an asymmetric PCR method, using synthetic oligonucleotides KG6 and KG16 as primers. Primers used in DNA sequencing are shown. Upper arrows indicate antisense strand primers and lower arrows indicate sense strand primers.

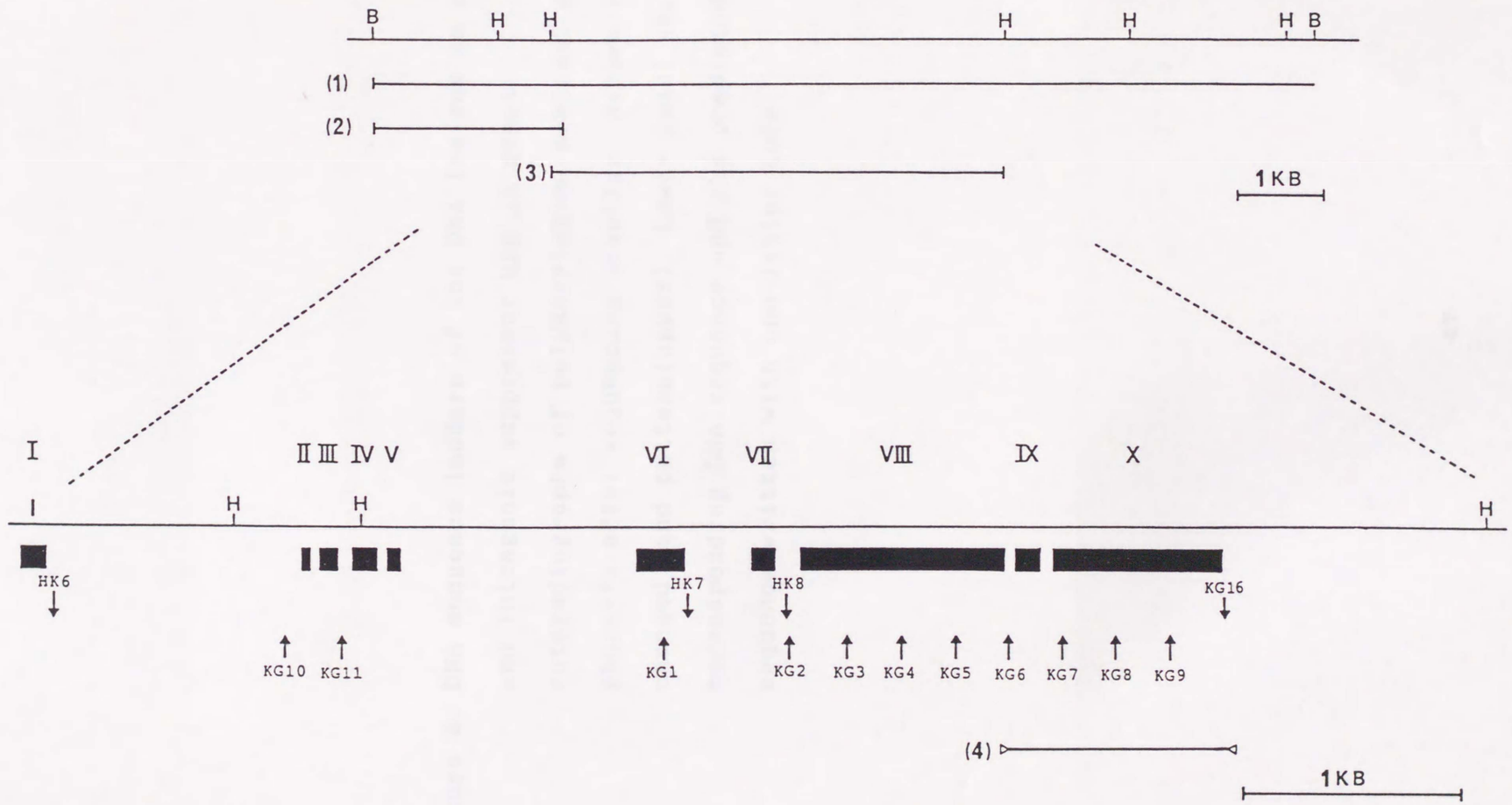
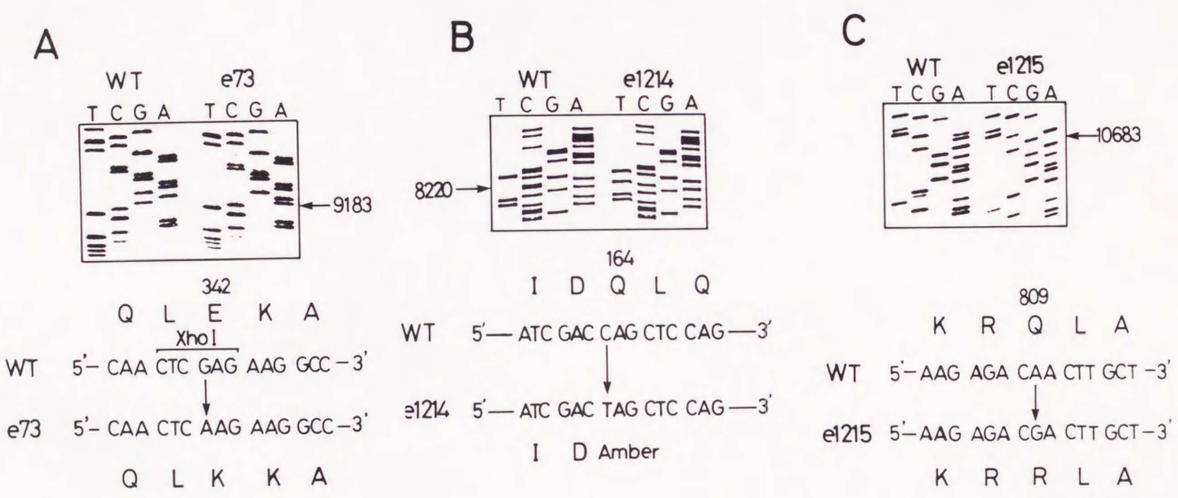

B
H
H
H
H
H
B
(1)
(2)
(3)
1KB
I
II III IV V
VI
VII
VIII
IX
X
I
H
H
H
HK6
HK7
HK8
KG16
KG10
KG11
KG1
KG2
KG3
KG4
KG5
KG6
KG7
KG8
KG9
(4)
1KB

Figure 9: DNA sequence ladders of the DNA lesions in the mutant and intragenic suppressor unc-15 genes.
Autoradiographs of polyacrylamide gradient gel electrophoresis after sequencing reaction. Arrows indicate the changed band pattern(upper). Lower panel show the corresponding DNA sequence and the predicted amino acid sequence written with one-letter code.


A
WT
e73
T C G A T C G A
9183
342
Q L E K A
Xho I
WT 5'- CAA CTC GAG AAG GCC -3'
e73 5'- CAA CTC AAG AAG GCC -3'
Q L K K A
B
WT
e1214
T C G A T C G A
8220
164
I D Q L Q
WT 5'— ATC GAC CAG CTC CAG —3'
e1214 5'— ATC GAC TAG CTC CAG —3'
I D Amber
C
WT
e1215
T C G A T C G A
10683
809
K R Q L A
WT 5'-AAG AGA CAA CTT GCT -3'
e1215 5'-AAG AGA CGA CTT GCT -3'
K R R L A

D

799

L T E K L

WT 5' —CTT ACC GAG AAG CTC—3'

e1402 5' —TTT ACC GAG AAG CTC—3'

F T E K L

E

835

D A E G R

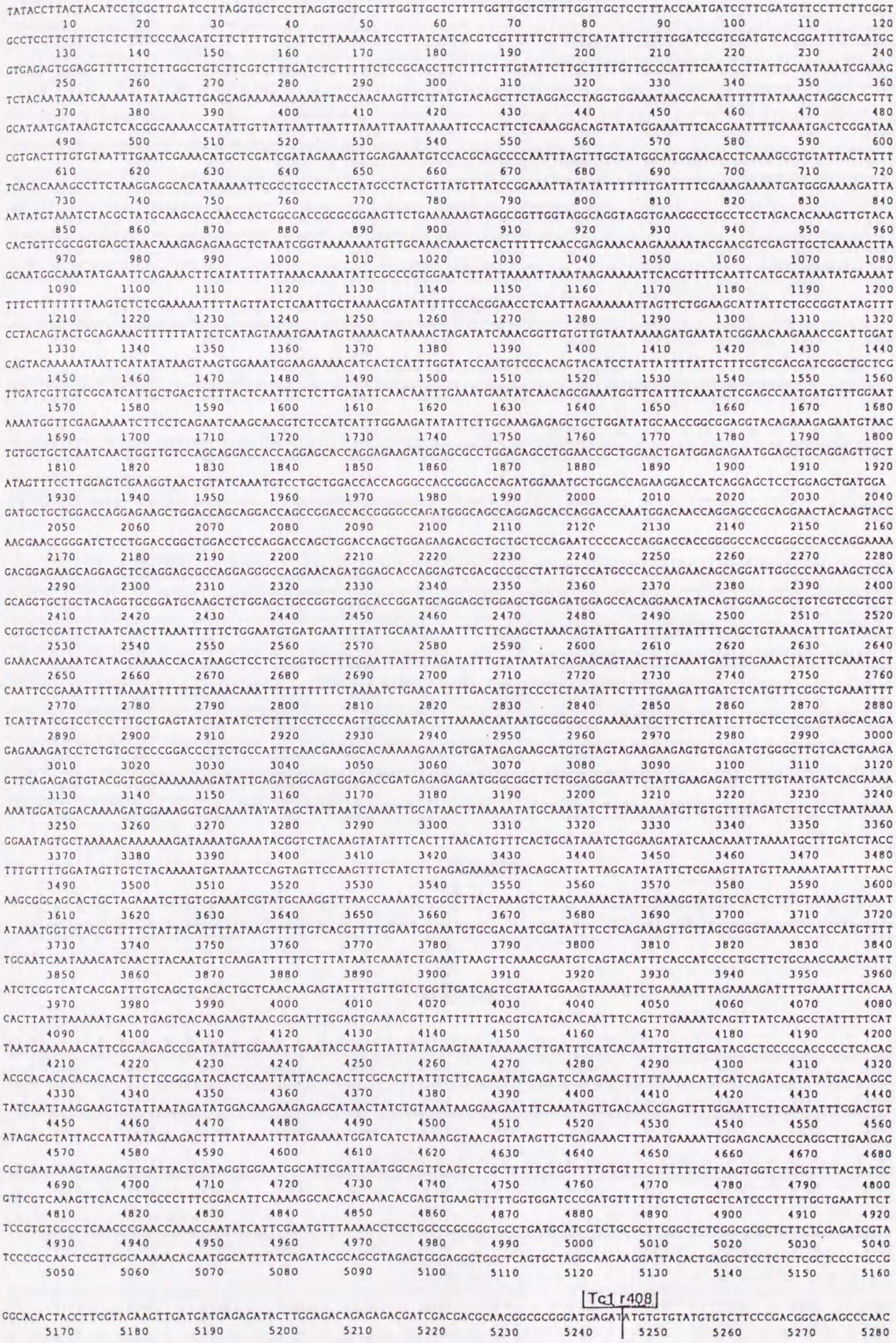
e73 5'— GTA GCT GAG GGC CTG—3'

e73m209 5'— GTA GCT AAG GGC CTG—3'

D A K G R

Figure 10: The mutations in the complete DNA sequence of *unc-15* gene

Nine *unc-15* mutations determined in this work are shown. DNA sequence of the gene has been previously reported(Kagawa *et al.*, 1989). Coding sequences have been translated using the standard 1 letter code. Upper arrows indicate the changed amino acid residues, and lower arrows indicate the changed nucleotides. Peptide sequences that correspond to sequenced peptides from paramyosin are boxed. The site of insertion of Tc1 in strain *r408* is also indicated(Kagawa *et al.*, 1989).


Tc1 r408

M S L Y R S P S A A L L K

GCACAATCAGTCAGTCTCGAAGCAGCCAGCCAGCCAGCAGCACACACAGGGGCACCATCCATTTGAATACTCCATTTGATCATGTCATTGTATCGTTCGCCATCCGCTGCTCTTCTTAAG
5290 5300 5310 5320 5330 5340 5350 5360 5370 5380 5390 5400

S P S Q A A F G A P F G S M S V A D L G S L T R L E

AGCCCGTCGCAAGCAGCGTTCGGTGCTCCATTTGGCAGCATGTCCGTCGCCGATCTTGGATCACTCACCAGACTCGAGGTAAATTTTTGAATGTTTTCTACCATTTCTGAACATTTCACT
5410 5420 5430 5440 5450 5460 5470 5480 5490 5500 5510 5520

GCCAAAACCCCAAAACCCGTGTTCCATTTTTCATCGAATCGATGTTTCATTGACTGATATTTTGAATTATATCTGTAATTTAAATTTTAGTAAAAAAGGCATCTAATATAGCAACGAAGA
5530 5540 5550 5560 5570 5580 5590 5600 5610 5620 5630 5640

ATTGAAAAACTTGAAAAATCTCAAATTGTAGTTTTACAGTACTCCTCAGTCTCAAGAAAAATGTTTGTTTTGTCCCTAAATTGAATGATTGACAAATAACGGCGTTTTCAGTTTTGTAAA
5650 5660 5670 5680 5690 5700 5710 5720 5730 5740 5750 5760

TATTATTTTTCTGCATCAAAATGTATGGAATAAAACAATATTCCGAGCCAAAACACTATTTTCAGAAAAATTTTTAAACTTCCTAGTTTTCTCTATAATTAAACCTTTAGATTCCTTATT
5770 5780 5790 5800 5810 5820 5830 5840 5850 5860 5870 5880

CTAAATCTTCATTTTGAAAAATCTGTTCATCATTCATCTCGGCAACTAGTTTCTTTGTGTAACTAAGATTTGCAACCTTTGTTTAACTTACTGCTTGCTTACATATATTTCCAAGAAAAA
5890 5900 5910 5920 5930 5940 5950 5960 5970 5980 5990 6000

GTAGAACCCTAATTCTCAGATTTTCAAATATAGCACTTATAAAAAACAAAAATTACGCTTTTTTTCTTTGAAAAAACAATTATTTTTTGACCCCATATTTTCTCATGGTTCTAGCTACTG
6010 6020 6030 6040 6050 6060 6070 6080 6090 6100 6110 6120

AATTTGCGATTATTGCCAATTGCCTGCAAAAACATTTTGTCAATTAAAATAAAAACAATTACCAAATCATTCGCTGGTCTATTTCCAAAAAATGCACTCTACGTCTCTCATATCTATAAG
6130 6140 6150 6160 6170 6180 6190 6200 6210 6220 6230 6240

CTATTTTCACGTTTCTCTTATAATCTCCCACACTGTGTTGTAAAATTTCGAAGAAACCTAATGCATTATTTGTTAAGCTTAGATATCAATTCAAAAAAAAAACTAAATGGTAATATCGAA
6250 6260 6270 6280 6290 6300 6310 6320 6330 6340 6350 6360

TTGTATGCTACGAGTGTATTCTTGTCGTTTGATCTCAAGAAATTCCGGGACATTTAAATTTGTGACGTCAGGATGTTTATCTTCACAGTACTTTAAAAATATTCCCCACTCGGCACTAAA
6370 6380 6390 6400 6410 6420 6430 6440 6450 6460 6470 6480

TTTATAGGTCCGTGTAATAAGGTTCATCAAGAAGTTCAATCAGAATCGGAATCCATACTTTTTTTATCTGAATCAGTTCTTGAAACAATTAATTCTTCAAAGATTTTACAAATTAATACA
6490 6500 6510 6520 6530 6540 6550 6560 6570 6580 6590 6600

D K I R L L Q E D L E S E R E L R N R V

GTTTCACTTTTCAGGACAAGATCCGTCTTCTCCAAGAGGATTTGGAATCAGAGCGCGAACTCCGCAACAGAGTGAGTTTTTACCTTTTATCTCTGATACCATGATTGCCATTTTCAGGTT
6610 6620 6630 6640 6650 6660 6670 6680 6690 6700 6710 6720

E R E R A D L S V Q V I A L T D R L E D A E G T T D S Q

GAACGTGAGCGTGCCGATCTCTCCGTTCAAGTTATTGCTTTGAGTGACAGACTCGAGGATGCCGAGGGAACCACCGACAGCCAAGTGAGTTTCCAGTTTTTATGAATTTCAATTTAAAGA
6730 6740 6750 6760 6770 6780 6790 6800 6810 6820 6830 6840

I E S N R K R E G E L S K L R K L L E E S Q L E S E D A M

AACATCTTATACCTTTAAACATTATAATTACAGATCGAATCGAACCGCAAACGTGAGGGAGAACTCTCAAAGCTTCGCAAGCTCCTCGAGGAGTCTCAACTTGAATCCGAAGATGCCATG
6850 6860 6870 6880 6890 6900 6910 6920 6930 6940 6950 6960

N V L R K K H Q D S C L D Y Q D Q I E Q L Q K K

AATGTTCTCCGCAAAAAGCACCAGGTACAGTAATCTTTATGCTATAGCTTAAAGAATTCGTGAAATTTTAGGATTCCTGCCTCGACTACCAAGATCAGATCGAGCAACTCCAAAAGAAAA
6970 6980 6990 7000 7010 7020 7030 7040 7050 7060 7070 7080

N A K

ATGCCAAGTAAGTTATTATCTTTATTTTCTAAGTTTTTTTTCGTATTAGTACTTGTGCCTTCATTTTCTTCTTCTTCTTCTATCTTCCCGCGTTGAAATGGCAGCGGAGGAGGTTGGC
7090 7100 7110 7120 7130 7140 7150 7160 7170 7180 7190 7200

GGCTCGGCAGAAGCTCTGAAGAAGAAGTATAAGAAGATGAAGAAGCATCAGCTCAAAGTGTGTAAAATAACAAACGTTTCTCGTGTGTGTGTGTTTGTTTCGCCTTCCAGGAAGAGATGA
7210 7220 7230 7240 7250 7260 7270 7280 7290 7300 7310 7320

GAGAGATACAAGAATGTCTGCGTCTTACTGATCTTCTCAATATCTCTCACATTTCTCTCATTGATCTTGATGCTTGGCGAGCAATCTGCGTTCCCGTGAAACAGTGGTGGTGGTAAAAAA
7330 7340 7350 7360 7370 7380 7390 7400 7410 7420 7430 7440

TAATGAGATTAAGTTGGGATGAGAAGTTGAGATGAACGGGAAGGGGACAGCTTGAGAACTTGAATGACGAATGCATGAACATATTATAGAATATTTAAAAATGTAGTAAGGATCAAACAT
7450 7460 7470 7480 7490 7500 7510 7520 7530 7540 7550 7560

TATTTGTTTCATAACAAGGGGTTGCTAGCCTAGTCTTGCACCTCCTATCCTCTAGTTTTACAGCTCATATCTCGGTTATTTTTCATTTATGAACACATTTCAACAACAAAAATGCGTGAG
7570 7580 7590 7600 7610 7620 7630 7640 7650 7660 7670 7680

AAATCCTGTATATTAATAGGAAAAATACTGTATTTTTCCCATTTAAATTACGATGGATTCATTTTATGATGTCTCTTTTTTTTGCTCAAATAACTTTCAATGAAATCAAGGAAAATCTTC
7690 7700 7710 7720 7730 7740 7750 7760 7770 7780 7790 7800

CGTAATTTAAAACACATTTCTAGAATCTATGTTAATCTTTTAAACAATGTACATAGATGATCTATTCAGTTGTAAAATAAGTTTTAGTTGTTCCCTTCTTGCGTTCAAAAATCTTCGAGT
7810 7820 7830 7840 7850 7860 7870 7880 7890 7900 7910 7920

TCAACTTTCCCATGAAAATAATTGTGTGCAACTCTTGACGTAATGTTTTTAAATCCAAAATTAGTTTATACTTTTCCACCCACTATCAATAGTTTCACCTGTCCAAACCGGCTGCTTTCT
7930 7940 7950 7960 7970 7980 7990 8000 8010 8020 8030 8040

GACATCATTCATAGCCAAACATATTGTTGACACATCAAGAATTACTTGTTCGCCGCTGCTTGACTTTTTGTTAAATTCCAAATATCCTCCCATCCCACGCAATATCATCATCATTCAGGA
8050 8060 8070 8080 8090 8100 8110 8120 8130 8140 8150 8160

Amber

I D R E R Q R V Q H E V I E L T A T I D Q L Q K D K H T A E K A A E R F E A Q A

TCGACCGCGAACGCCAACGCGTCCAACACGAAGTCATCGAGTTGACCGCCACTATCGACCAGCTCCAGAAGGACAAACACACCGCTGAGAAGGCCGCTGAGCGCTTCGAAGCCCAAGCCA
8170 8180 8190 8200 8210 8220 8230 8240 8250 8260 8270 8280

e1214

N E L A N K V E D L N K H V N D L A Q Q R Q R L Q A E N N D L L K

ACGAACTTGCCAACAAGGTCGAGGACTTGAACAAGCACGTCAATGACCTCGCCCAACAACGTCAACGCCTTCAAGCTGAGAACAATGATCTTCTCAAGGTTTGTTTTTTTGAAGTATCATG
8290 8300 8310 8320 8330 8340 8350 8360 8370 8380 8390 8400

TGTATAATCTCTAGTTTTATCATCTTTATCACGTGCCACCGATCACTCTGCTCATCTTTCTACTTTTTTTTAGCAAAAAAATGCGGTAAAAAGAAAAAAAGAAACCCCTTTTAACAGGGG
8410 8420 8430 8440 8450 8460 8470 8480 8490 8500 8510 8520

GTTTCTTTACTAAATTCTCTATTTTATCGCTACTATTTCCGCAGAGTGTGTGTGCAAATAGAGTACTCGAGATATTAGAAAGGAGGATGCTCTGTGACGCTCATCGTTTTTTTGTCTCGT
8530 8540 8550 8560 8570 8580 8590 8600 8610 8620 8630 8640

E V H D Q K V Q L D N L Q H V K Y T L A Q Q L E E

TCAGTGTGTTCTGTTAGCAGATCAGGGATCGTTAATGTTTTTCAGGAGGTTCACGACCAGAAGGTTCAACTCGATAACCTTCAACACGTCAAGTACACCCTCGCTCAACAGCTCGAGGAG
8650 8660 8670 8680 8690 8700 8710 8720 8730 8740 8750 8760

A R R R L E D A E R

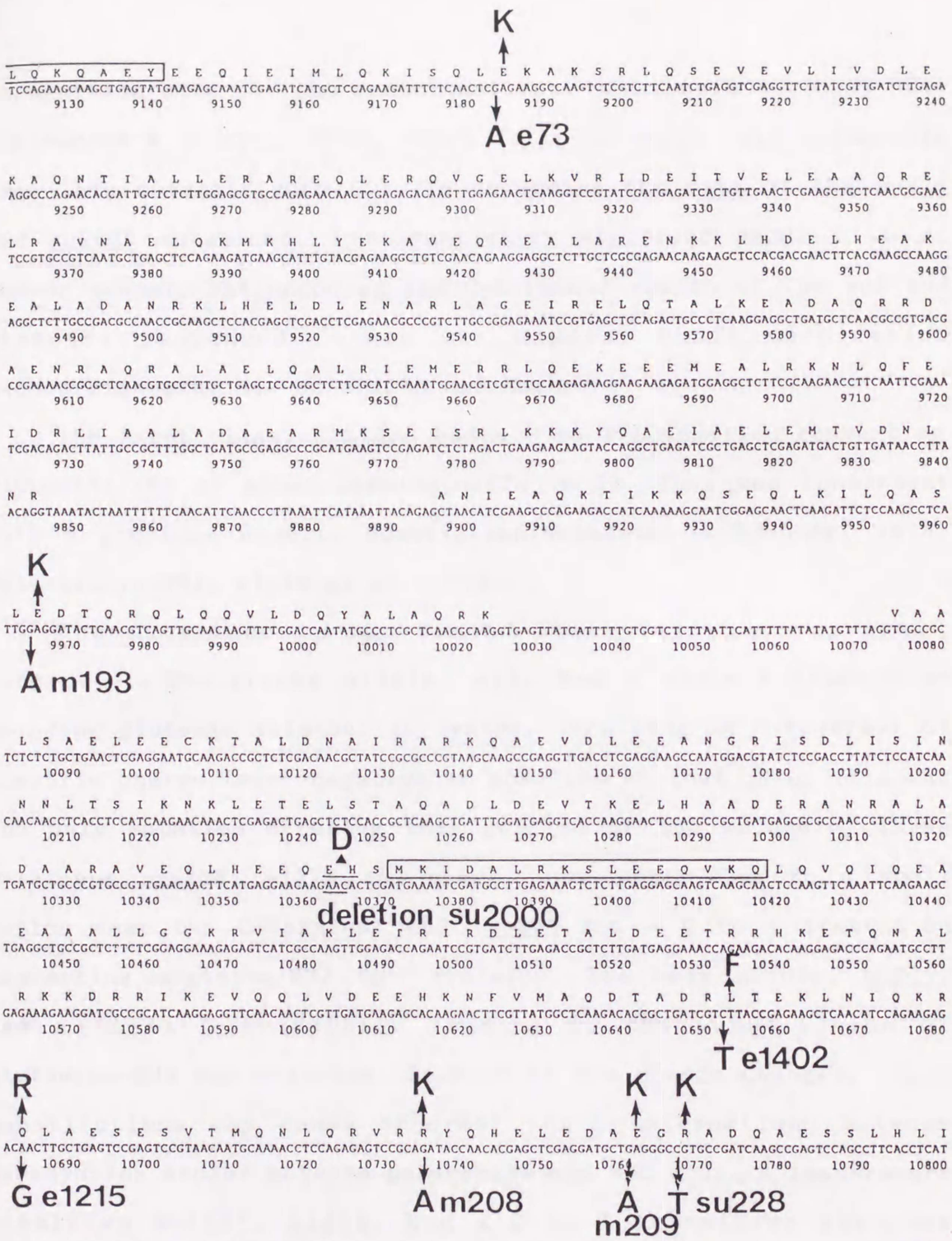
GCCAGACGTCGTCTTGAGGATGCCGAGCGCGTAAGTCTACTAGTCCAAAGTGCCAAATTCAGAGGCGGAAATATCGGGTTTCTTCCTCTGACGCATTTCTGGACATTAAAAAAGCATAAA
8770 8780 8790 8800 8810 8820 8830 8840 8850 8860 8870 8880

E R S Q L Q S Q L H Q V Q L E L D S V R T A L D E E S I A

CGTGCGAAGATCAAAGAACGTTGAAATTTCAGGAGCGCTCCCAACTTCAATCTCAACTCCACCAGGTTCAGCTCGAGCTCGACTCGGTCCGCACCGCTCTCGATGAGGAGTCGATTGCCC
8890 8900 8910 8920 8930 8940 8950 8960 8970 8980 8990 9000

R S D A E H K L N L A N T E I T Q W K S K F D A E V A L H H E E V E D L R K K M

GTTCCGATGCTGAGCACAAGCTCAACCTTGCCAACACTGAGATCACCCAGTGGAAGAGCAAGTTCGATGCTGAGGTCGCCCTTCACCACGAAGAAGTTGAGGATCTCCGCAAGAAGATGC
9010 9020 9030 9040 9050 9060 9070 9080 9090 9100 9110 9120


K
A e73
K
A m193
D
deletion su2000
F
T e1402
R
K
K
K
G e1215
A m208
A m209
T su228

recognition site for the monoclonal anti-paramyosin antibody NE1-6B2(Kagawa & Gengyo, 1988), which fails to react with paramyosin from this mutant(Figure 14). To determine the site of the su228 and su2000 mutations, I enzymatically amplified about 1 kb of mutant genomic DNA encoding the C-terminal region of the rod and directly sequenced, using the dideoxy chain termination method(Figure 8: See Materials and Methods).

The first class, e1214, had a C to T transition converting glutamine-164 to amber terminator(Table 2). This was consistent with a previous genetic observation(Waterston & Brenner, 1978; Waterston, 1981; Wills et al., 1983).

The mutations in the second class fell into several subgroups. The strong allele, e73, had a G to A transition changing glutamic acid-342 to lysine, resulting in a reversal of electric charge from negative to positive at that site. This was the only mutation altering this portion of the molecule. Three mutations, su228, e1215 and e1402, were restricted to a small region near the C-terminal end. su228 had a C to T transition converting arginine-837 to cysteine. The weak allele, e1215, had an A to G transition leading to the substitution of glutamine-809 for arginine. Because of the charge changes, these substitutions may cause abnormal ionic interactions between paramyosins and/or between paramyosin and MHC rods. A temperature sensitive mutant, e1402, had a C to T transition changing leucine-799 to phenylalanine. This substitution may destabilize the α -helical coiled-coil structure due to the bulk of the phenylalanine side chain.

The last mutation of this class, su2000, was not a simple

Table 2: The mutations of *unc-15* alleles and *unc-15* suppressor alleles.

Numbers in the mutation column indicate the position of the mutations in the nucleic acid sequence(numbered according to Kagawa *et al*., 1989), with the changed bases circled. Numbers in the amino acid substitution column indicate the location of the changed residue within *unc-15* paramyosin, with the N-terminal methionine as residue 1. Mutant phenotypes were summarized from the original papers(Waterston *et al*., 1977; Rose & Baillie, 1980).

	Allele	Phenotype	Mutation	Amino acid substitution
unc-15 mutants				
	e1214	paralyzed	CAG→TAG 8220	Q→Amber 164
	e73	severely uncoordinated	GAG→AAG 9183	E→K 342
	e1215	weakly uncoordinated	CAA→CGA 10683	Q→R 809
	e1402	temperature sensitive 16°C:wild type 20°C:uncoordinated 25°C:sterile	CTT→TTT 10652	L→F 799
	su228	severely uncoordinated	CGT→TGT 10766	R→C 837
	su2000	severely uncoordinated	AAC→deletion 10362-10364	EH→D 702-703
intragenic revertants				
	e73m193	pseudo-wild type	GAG→AAG 9964	E→K 586
	e73m208	pseudo-wild type	AGA→AAA 10734	R→K 826
	e73m209	pseudo-wild type	GAG→AAG 10760	E→K 835

Figure 11: Localization of mutations of the unc-15 gene.
Upper; Genetic map reported previously by Rose & Baillie(1980). However, the orientation of the genetic map has been reversed to fit the physical map obtained from our molecular work. Lower; The sites of the nine unc-15 mutations are shown. The location of six unc-15 mutants are indicated above the bars and the four unc-15 alleles that have been genetically mapped are starred. The alleles isolated as internal suppressors of e73 are shown below the bars.

Genetic Map
unc-13
e1214
e73
su2000
su228
1 x 10⁻³ mu
unc-15 Gene
e1214
e73
m193
su2000
e1402
e1215
m208
m209
su228
1kb
unc-13

missense change, but instead three bases AAC(11362-10364) were deleted, leading to the substitution of a single aspartic acid residue for the glutamic acid-histidine pair(702, 703). Removal of an amino acid might seriously perturb coiled-coil structure locally.

For the three internal revertants of e73: m193, m208 and m209, I cloned and sequenced all exons of the unc-15 gene from e73m193, e73m208 and e73m209. The mutations m208 and m209 were located in the C-terminal portion, and were G to A transitions changing lysine-826 to arginine and glutamic acid-835 to lysine, respectively. The weakest revertant, m193, had a G to A transition changing glutamic acid-586 to lysine(Table 2).

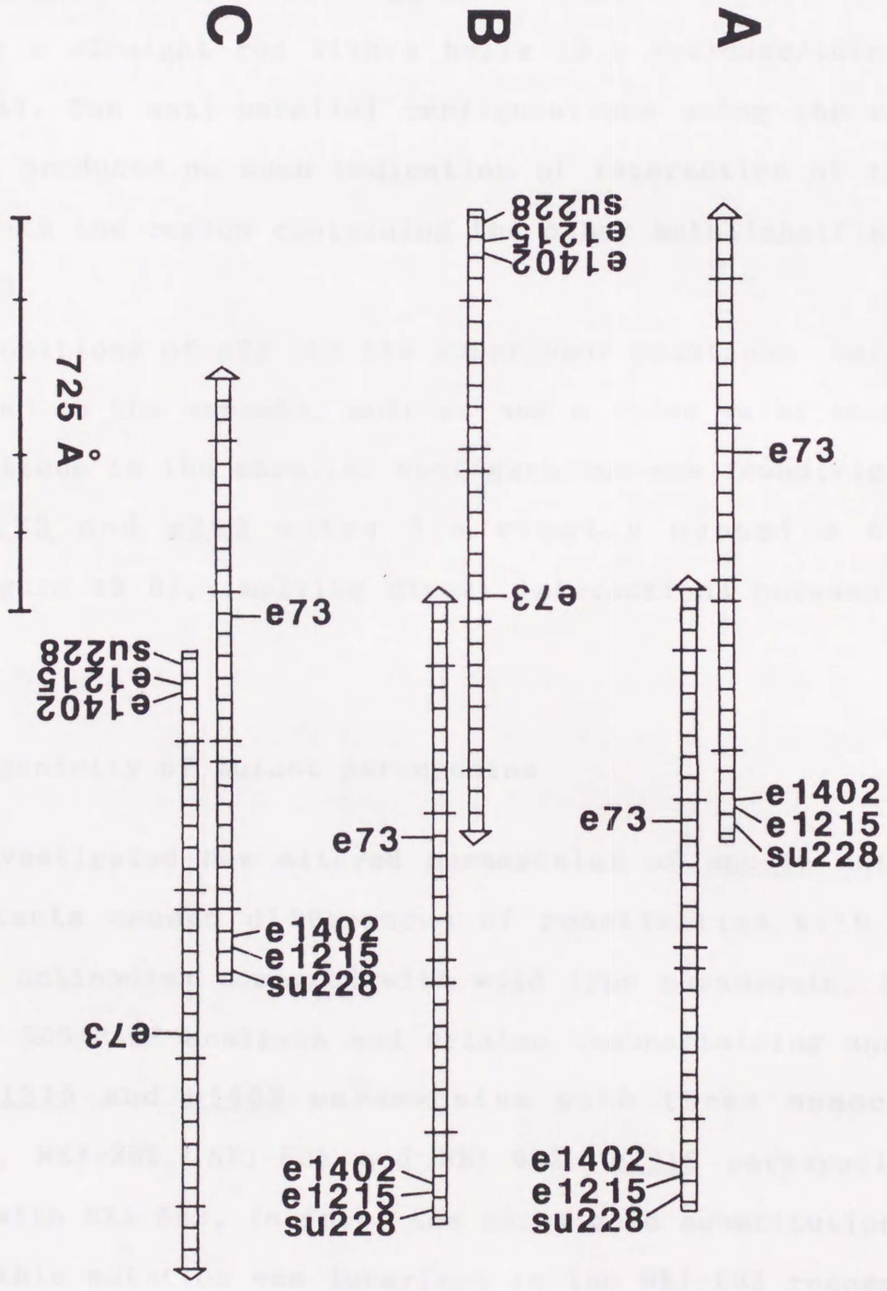
Figure 11 shows genetic allele map of unc-15 alleles reported previously by Rose & Baillie(1980) and physical map of the mutations of unc-15 alleles and suppressor alleles. The coincidence of the orientation of the unc-15 gene in the genome between these maps is discussed(see discussion).

4.3 Relationships between mutations and molecular models of paramyosin assembly

Previously Kagawa et al. have constructed models of the possible arrangement of paramyosin rods in pairwise combinations, with the rods either in the same orientation(classically called "parallel") or in opposite orientation ("anti-parallel")(Kagawa et al., 1989). In the opposite orientation, either the N-termini(anti-parallel NN) or the C-termini(anti-parallel CC) may be overlapping. To interpret the effects of the substitutions on

Figure 12: The relationships between the sites of missense mutations in the unc-15 gene and the proposed arrangements of paramyosin molecules assembled in the thick filament.

Paramyosin helical rods with residues 31 to 853 are drawn as open bars; arrowheads indicate the N-terminus. Short vertical bars indicate the spacing of the 28-residue repeats, long vertical bars indicate the position of skip residues, which periodically interrupt the 28-residue repeat(Kagawa et al., 1989). (A); Parallel overlap of paramyosin molecules with a 493 residue stagger. The overlapping region is 330 residues long. (B); Anti-parallel NN overlap with a 515 residue stagger. The overlapping region is 308 residues long. (C); Anti-parallel CC overlap of paramyosin molecules with a 358 residue stagger. The overlapping region is 465 residues long. The scale of 725Å is equivalent to 493 residues.


C
B
A
725 Å
e73
su228
e1215
e1402
e73
su228
e1215
e1402
e73
e1402
e1215
su228
e73
e1402
e1215
su228
e73
e1402
e1215
su228

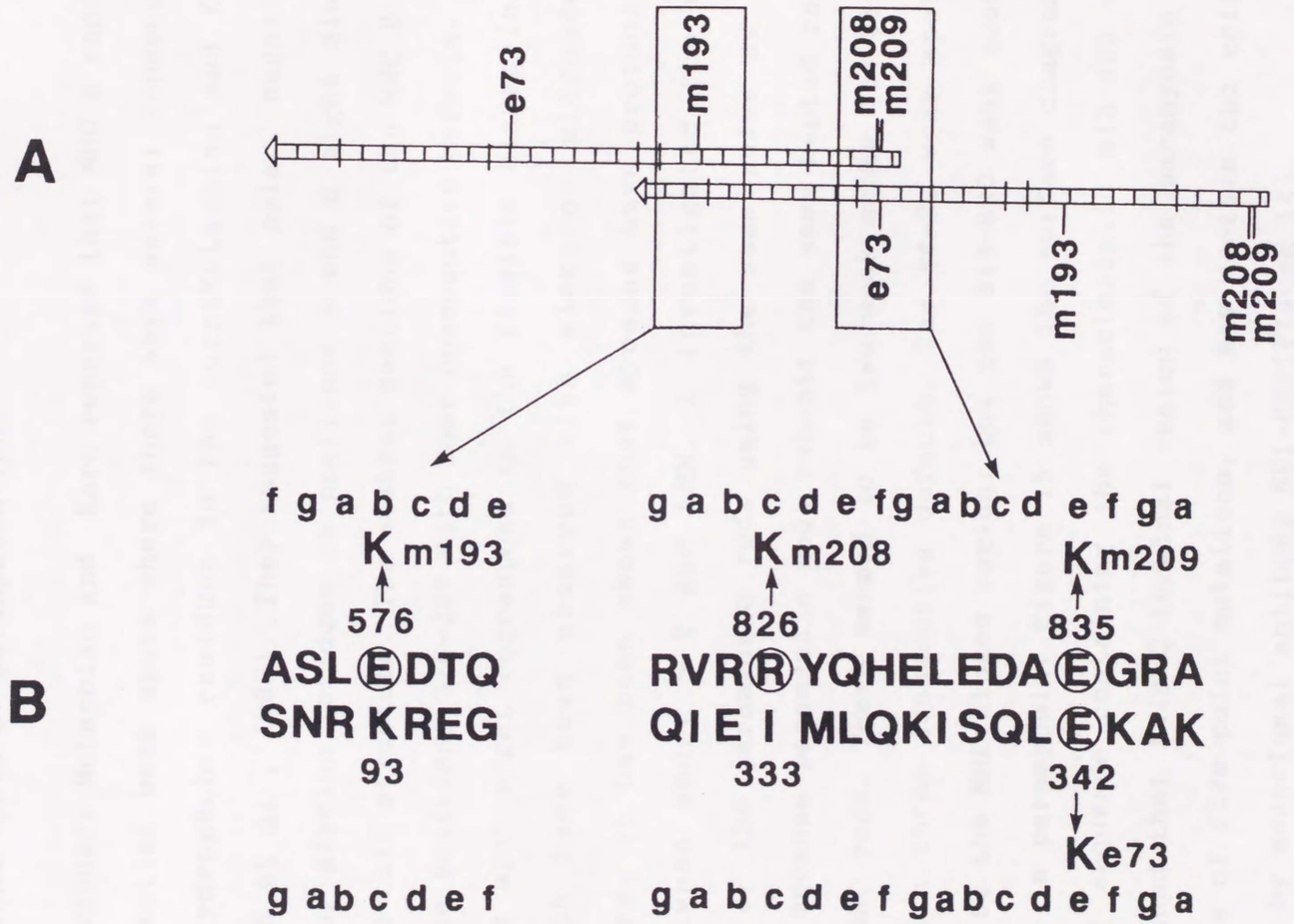
paramyosin assembly, I superimposed the missense mutations on the models(Figure 12). In the parallel configuration using the optimal stagger of 493 residues, the e73 substitution lies opposite the C-terminal region containing the other missense mutations(Figure 12 A; this stagger corresponds to an overlap of 725 Å, for a straight rod with a helix [3.5 residues/turn] rise of 1.485 Å). The anti-parallel configurations using the optimal alignments produced no such indication of interaction of the e73 mutation with the region containing the other mutations(Figure 12 B and 12 C).

The positions of e73 and its suppressor mutations were also superimposed on the assembly models, and a close relationship of their positions in the parallel configuration was found(Figure 13 A). The e73 and m209 sites lie exactly opposite to one another(Figure 13 B), implying direct interactions between these residues.

4.5 Antigenicity of mutant paramyosins

I investigated how altered paramyosins of unc-15 mutants and revertants caused differences of reactivities with three monoclonal antibodies compared with wild type paramyosin. Figure 14 A shows SDS-PAGE analysis and related immunostaining analysis of e73, e1215 and e1402 paramyosins with three monoclonal antibodies, NE1-2B5, NE1-5B5 and NE1-6B2. e1215 paramyosin did not react with NE1-6B2, in fact, the amino acid substitution(809) caused by this mutation was locarized in the NE1-6B2 recognition site(773-865)(Kagawa and Gengyo, 1988; Figure 14 B). Paramyosins

Figure 13: The spatial relationship between the site of the e73 mutation and the sites of the internal e73 suppressor alleles when paramyosin molecules associate as in the parallel model(Figure 12A).
(A); Parallel overlap of paramyosin rods with a 493 residue stagger. The mutation sites are indicated by vertical lines and the allele number. (B); The amino acid sequence of the boxed regions of panel A. The upper and lower lines of amino acid sequence correspond to the upper and lower paramyosin molecules diagrammed in panel A. Wild type residues changed in the suppressor mutations are circled and arrows indicate the substituted amino acids. a, b, c, d, e, f and g indicate the positions within the heptad repeat; hydrophobic residues are concentrated in the a and d positions. Note that the substituted amino acids of e73 and m209, suppressor of e73, lie directly opposite one another in the parallel model of paramyosin association. The possible mechanism of this suppression is discussed in the text.


A
e73
m193
m208
m209
e73
m193
m208
m209
B
f g a b c d e
K m193
576
ASL E DTQ
SNR KREG
93
g a b c d e f
g a b c d e fg a b c d e f g a
K m208
826
K m209
835
RVR R YQHELEDA E GRA
QI E I MLQKI SQL E KAK
333
342
K e73
g a b c d e f g a b c d e f g a

produced by other mutants and revertants, had no prominant changes of their reactivities(data not shown).

4.5 The weak spots of paramyosin rods

Previously McLachlan and Karn reported that MHC B rods had three potential weak spots where there were several consecutive missing hydrophobic residues in the core(McLachlan and Karn, 1982; Karn et al., 1985). They suggested that polar, negatively charged, or glycine residues in positions a and d might disturb the coiled-coil structure. The weakest sections of the MHC B rods seems to be positions 316-327 with four consecutive defects. This major weak spot might correspond to the flexible hinges in the rod, which have been observed with electron microscopy. Furthermore, it has been shown that myosins have proteolytic sites between short S-2 and LMM. I investigated the weak structure of the paramyosin rods using the same rules as used with MHC, because paramyosin rods exhibit the same heptad repeat found in MHC rods. There seemed to be two weak spots, 84-91 and 813-820 with three consecutive defects. The 84-91 weak spot is analogous to the MHC hinged region, but the 813-820 weak spot is found only in paramyosin. Figure 15 shows the surface diagram of the unc-15 sequence containing the characteristic 813-820 weak spot. I found that this C-terminal region of the paramyosin rod is the site of five point mutations, and may contain the epitope recognized by monoclonal antibody NE1-6B2(Figure 14).

Figure 14: Immunoreactivity of mutant paramyosins when stained with monoclonal anti-paramyosin antibodies. (A); Panel(a), total proteins from C. elegans stained with Coomasie-Blue after fractionation on 6% to 10% SDS/polyacrylamide gradient gels. Panel(b), Nitrocellulose filter immunoblot replica reacted with monoclonal mouse anti-C.elegans paramyosin NE1-2B5. (c), Immunoblot reacted with monoclonal antibody NE1-5B5. (d), Immunoblot reacted with monoclonal antibody NE1-6B2. For each panel: Lane 1, total protein extract of C. elegans, strain N2. Lane 2, e1214. Lane 3, e73. Lane 4, e1215. Lane 5, e1402. Note that e1215 paramyosin fails to stain with NE1-6B2, as indicated by the arrow. (B); The location of the epitope recognized by each monoclonal antibody was determined with expression plasmids(Kagawa & Gengyo, 1988; Kagawa et al., 1989). Coding sequences are drawn as shaded boxes, introns as gaps. The recognition sites of the monoclonal antibodies are indicated below the bars. The regions recognized weakly by NE1-5B5 are drawn as open bars; the restricted region(nine residues) that is specifically recognized is drawn as a filled bar. The mutation site of e1215 was localized to the NE1-6B2 recognition site, as indicated with an arrow.

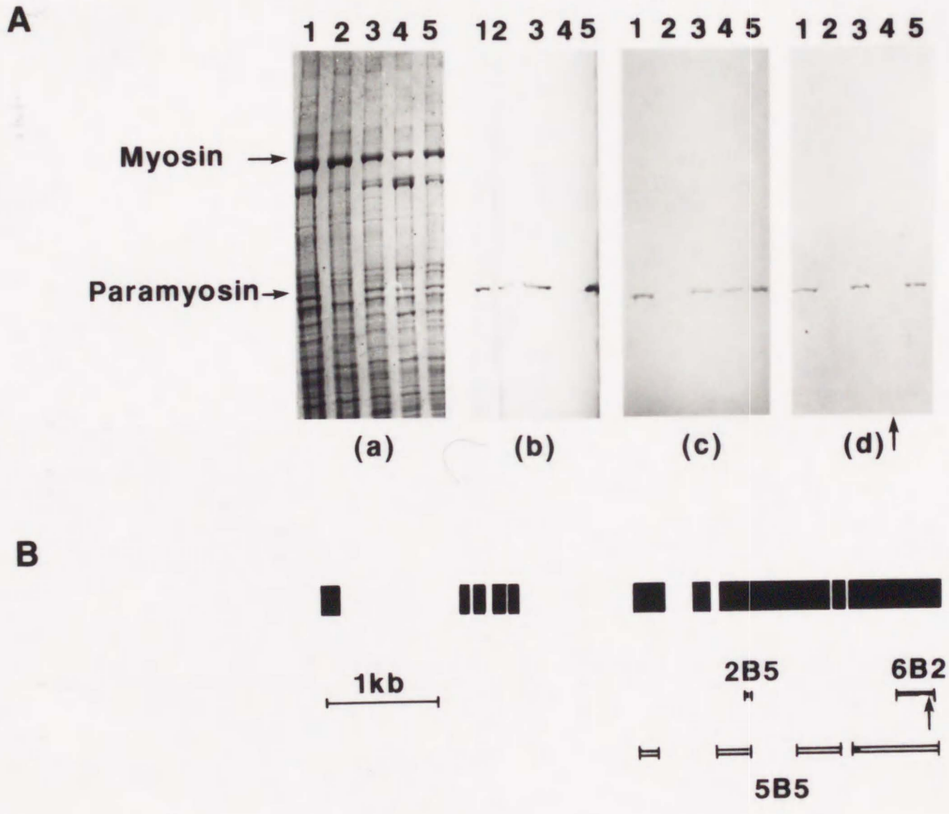
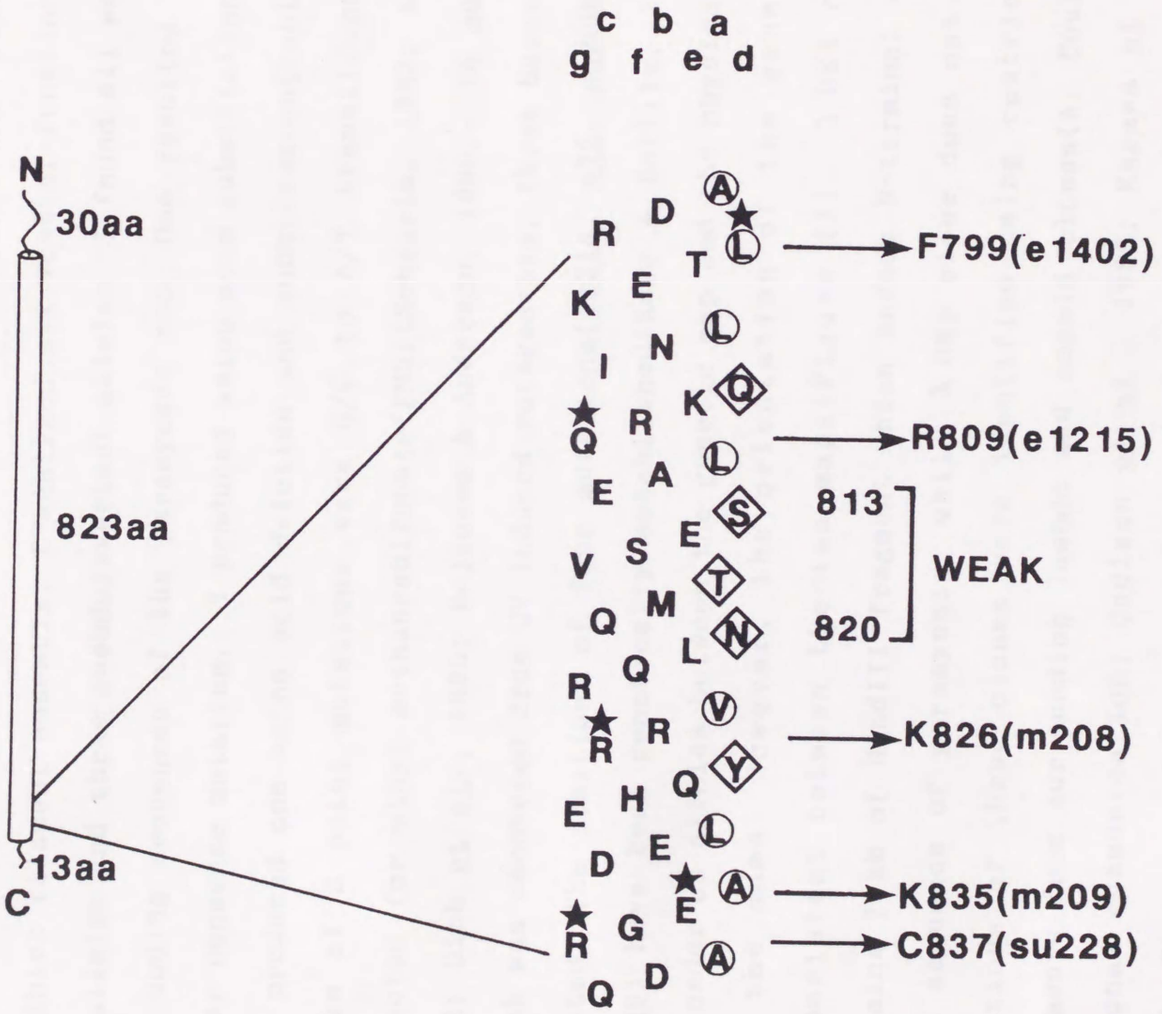

A
1 2 3 4 5
12 3 4 5
1 2 3 4 5
1 2 3 4 5
Myosin
Paramyosin
(a)
(b)
(c)
(d)
B
1kb
2B5
6B2
5B5

Figure 15: Coiled-coil surface diagram of the C-terminal region of unc-15 rod sequence from alanine-796 to glutamine-840.

The paramyosin molecule is schematically drawn on the left, with the N- and C-terminal non-helical regions and the rod of 823 residues represented. A portion of the rod near the C-terminus is enlarged on the right. The sequence is shown as a cylindrical projection of the outside surface of the α-helical chains. The sequence is read from right to left with a slight downward slant and is drawn with a pitch of 3.5 residues per turn. Residues with hydrophobic side chains located in the core positions a and d are circled. Diamonds enclose a- or d- position residues having acidic, polar or glycine side chains. These residues are likely to weaken the core. Stars indicate amino acid residues changed by mutations and arrows indicate the substituted amino acids. The region from 813 to 820 may be the short weak section of the paramyosin rod.

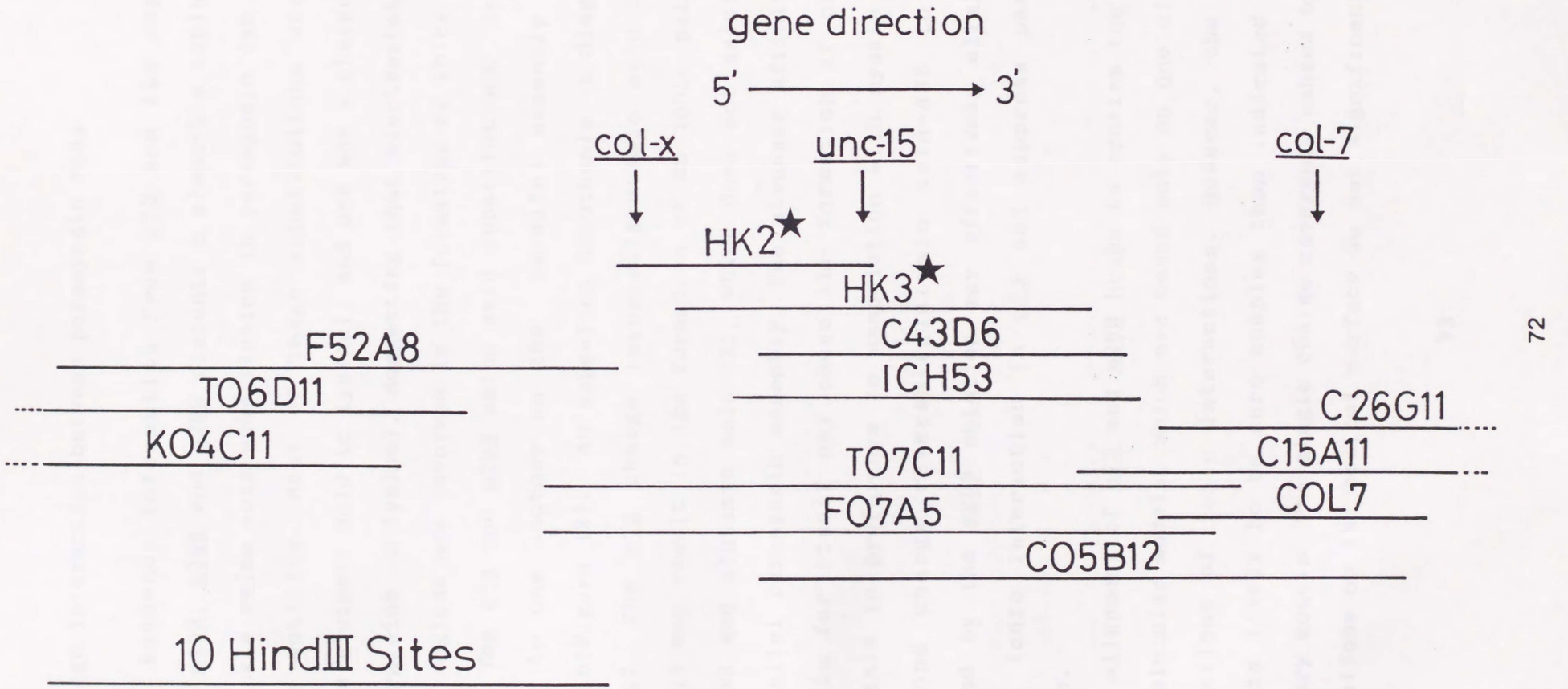

c b a
g f e d
N
30aa
823aa
13aa
C
F799(e1402)
R809(e1215)
813
WEAK
820
K826(m208)
K835(m209)
C837(su228)

5. Discussion

5.1 Mutations in the unc-15 gene

For the purpose of understanding the relationships between the amino acid sequence and the function of paramyosin molecules in thick filament assembly, I analyzed six loss-of-function unc-15 alleles and three pseudorevertant alleles. I found all were in the coding sequences of the paramyosin rod. One resulted in an amber nonsense mutation, 7 produced amino acid substitutions and one produced one amino acid deletion and substitution(Table 2). Seven of 8 point mutations were G/C to A/T transitions, as expected for ethyl methanesulfonate(EMS)(Schwartz, 1963; Krieg, 1963; Dibb et al., 1985; Bejsovec & Anderson, 1990). In su2000, which was recovered from UV induced mutagenesis, three bases were deleted. The positions of four mutations(e1214, e73, su2000 and su228) have been genetically established(Rose & Baillie, 1980). The order of alleles between the genetic map and the physical map was the same. However, the orientation of the gene was inconsistent between the two maps(Figure 11). λ HK2 clone contains 7 kb of HindIII fragment which encode N-terminal amino acid sequence of paramyosin, while λ HK3 clone does not. The locations of these clones were identified using restriction fragments from surrounding lambda and cosmid clones(A. Coulson, personal communication; Coulson et al., 1988; Kagawa et al., 1989). These results suggest 3' end of the unc-15 gene is near the col-7 gene(Figure 16). This inconsistency between the genetic and physical maps remains to be resolved.

Figure 16: Contig map of the region surrounding unc-15. Cosmid and bacteriophage λ clones covering unc-15 locus are represented. The λ clones HK2 and HK3 asterisked were used to analyze the sequence of the unc-15 gene. The locations of HK2 and HK3 on the physical map were done by matching "finger-prints" of these clones to the physical map database by A. Coulson. Two collagen genes, col-x and col-7, exist in the upstream and downstream of the unc-15 gene.

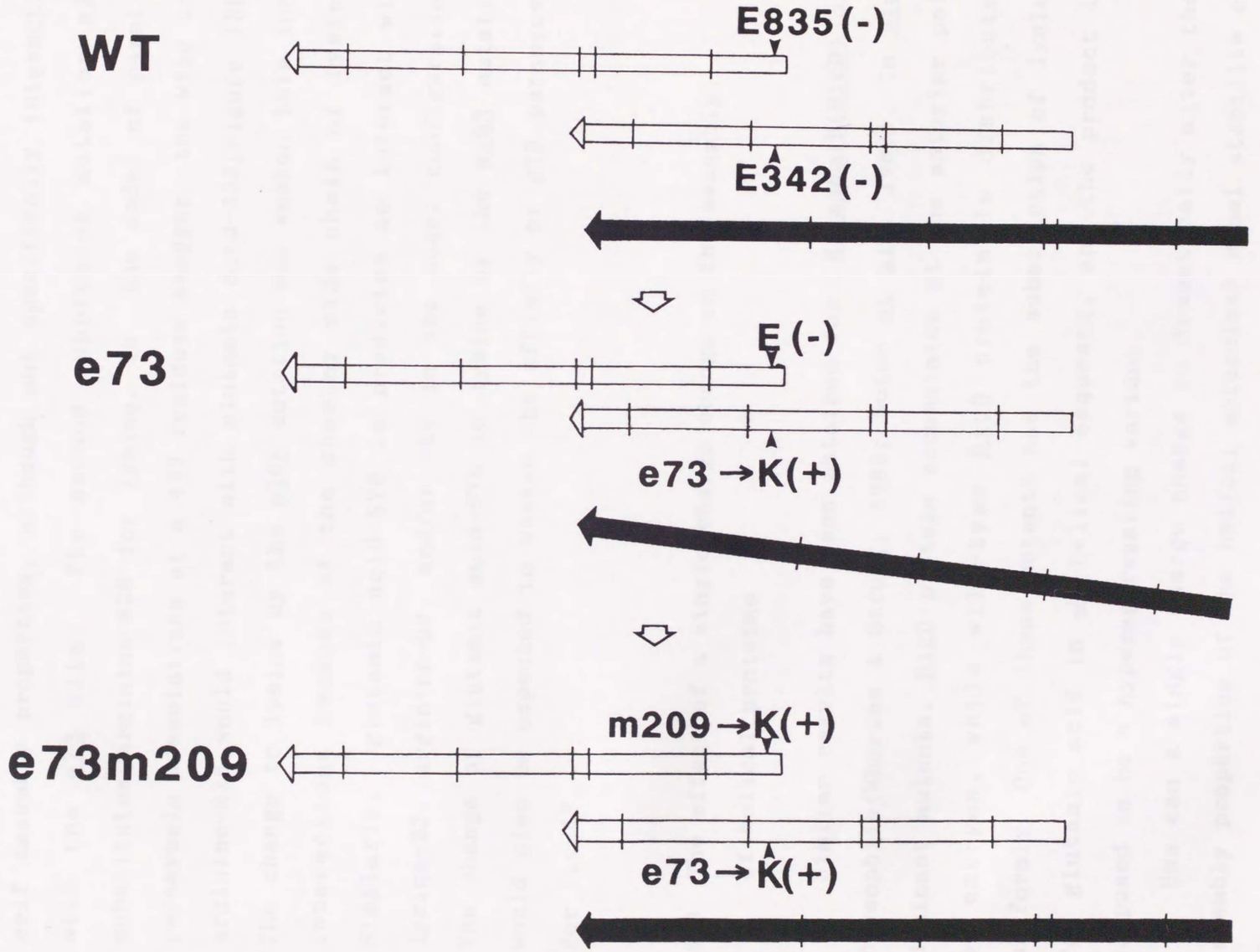

gene direction
5'
3'
col-x
unc-15
col-7
HK2★
HK3★
C43D6
ICH53
F52A8
TO6D11
KO4C11
C26G11
C15A11
TO7C11
COL7
FO7A5
CO5B12
10 HindIII Sites

5.2 Charge interaction between paramyosin rods

My sequence information from e73 and its suppressor alleles, m193, m208 and m209 presents a plausible explanation of how a single amino acid substitution in paramyosin can affect an animal's motility. Most of these substitutions were charge reversals(glutamic acid to lysine), and one was a charge strength change(arginine to lysine), suggesting that electrostatic forces in these regions are involved in the formation of thick filaments *in vivo*. The e73 and m209 amino acid substitutions lay exactly opposite to one another on the parallel assembly model of paramyosin(Figure 12). An appealing hypothesis is diagrammed in Figure 17. The e73 charge reversal[glutamic acid-342(-) to lysine(+)] may result in the formation of an ionic pair between lysine-342 and glutamic acid-835, which does not exist in wild type parallel paramyosin assembly. The stronger affinity of e73 paramyosin for itself may cause the formation of paramyosin paracrystals in preference to association with myosin *in vivo*. The second charge reversal(glutamic acid-835 to lysine) introduced by the m209 mutation may effectively eliminate the abnormal ionic interaction in e73 and suppress paracrystal formation.

The alignment of e73 and m209 helps to confirm the validity of our molecular models, which are based only on one dimensional considerations of ionic interactions. However, the assembly problem is likely to be more complex than indicated by these rudimentary models. The single charge reversals caused by e73 and m209 mutations on the helical surface do not significantly alter

Figure 17: Thick filament assembly *via* ionic interactions in wild type N2, *unc-15* mutant *e73* and *e73* intragenic revertant *e73m209* animals. *unc-15* paramyosin rods(31-853) are indicated as open bars, and *unc-54* myosin heavy chain rods are indicated as filled bars. Vertical bars indicate the positions of the skip residues. The effects of single amino acid substitutions in thick filament assembly were considered. In the *e73* mutation, a glutamic acid-342 was changed to a lysine; this introduces a positive charge that may lead to an ionic pair between lysine-342 and glutamic acid-835 of an adjacent paramyosin molecule. The resulting stronger affinity among paramyosin molecules may influence the interaction between paramyosin and myosin heavy chain required for thick filament assembly. The substitution of glutamic acid-835 for lysine in the intragenic suppressor mutation *m209* introduces a second positive charge and thereby eliminates the abnormal ionic pair possible in the *e73* animal.


WT
E835(-)
E342(-)
e73
E(-)
e73→K(+)
e73m209
m209→K(+)
e73→K(+)

the total interaction scores in our calculations, but in fact, they dramatically affect the assembly *in vivo*.

The other suppressor mutations probably alter paramyosin self assembly properties, although not specifically interacting with the *e73* site. The second suppressor mutation *m208* substitutes arginine-826 for lysine. In the model of parallel paramyosin associations at a 493 residues stagger, the wild type arginine-826 would interact with glutamic acid-332(Figure 12B). The change to lysine by the *m208* mutation may weaken this ionic interactions because of the shorter side chain of lysine. Similarly, glutamic acid-576 is predicted to interact with lysine-93, arginine-92, and/or -94 in the model configuration. The change of glutamic acid-576 to lysine by the *m193* mutation would also be expected to weaken the affinity of *e73* paramyosin for itself.

5.3 The effect of a single charge change on the assembly of helical proteins

Similar results have been obtained for *BicaudalD*(*BicD*) of Drosophila(Wharton & Struhl, 1989; Suter *et al*., 1989). In *BicD* dominant mutants, *BicD* protein accumulates at the anterior pole of embryos, while wild-type *BicD* protein is distributed uniformly. One of these mutants has the substitution of lysine for glutamic acid in α-helical sequence, and the product is supposed to be a hyperaggregating variant.

How can a single charge change so dramatically alter the assembly properties of the helical molecules? Final stability of

the assembling molecules is probably determined by many intra- and inter-molecular interactions of charged or hydrophobic residues so that a change in any one residue should only have minimal effect. However, many phenomena in biological systems are controlled by transient, sequential reactions. If a change occurs in an early step, any subsequent steps will be affected. In my case, the C-terminal region and the site with which it interacts may be involved in the initial steps of parallel paramyosin assembly. The charge reversal in e73 may accelerate or stabilize the initiation step of parallel paramyosin assembly, and result in the formation of paramyosin paracrystals in vivo. A second possibility is that paramyosin molecules require a certain distribution of electrostatic forces to stabilize the final state of the assembly. In either case the regions defined by the mutations e73 and m208, m209 are especially important in parallel assembly.

5.4 The C-terminal cluster may play an important role in thick filament assembly

All mutations sequenced in this report were contained within the rod structure, and five missense mutations(e1402, e1215, su228, m208 and m209) were localized to a small region(799-837) of the C-terminal portion. This C-terminal cluster coincides with a characteristic weak spot(813-820) where coiled-coil structure may be disturbed(Figure 15). The point mutant e1215(glutamine-809 to arginine), which is very close to the weak spot, fails to

stain with monoclonal antibody NE1-6B2(Figure 14). Perhaps the subtle differences of paramyosin structure in the C-terminal cluster may affect the antigenicity of paramyosin molecule. This region may also be important in thick filament assembly *in vivo*.

5.5 Interaction with other proteins

My results have suggested that in *e73*, altered paramyosin affinity for itself in parallel paramyosin assembly is a plausible explanation for the observed disruption of thick filament assembly. Of course, the *e73* mutation may also affect the association of paramyosin either directly or indirectly with myosin heavy chains and/or other muscle proteins. Paramyosin is thought to interact with MHC A at the center of the thick filament where polarity of the filament reverses. Genetic interactions between *e73* and *myo-3*, the structural gene for MHC A, have been demonstrated. Duplications of the *myo-3* locus(Maruyama *et al.*, 1989) suppress the *e73* phenotype, partially restoring motility(Riddle & Brenner, 1978; Brown & Riddle, 1985; Otsuka, 1986). Presumably, increasing the accumulation of MHC A 2- to 3- fold enables *e73* paramyosin to interact successfully with MHC A perhaps as a result of mass action. In future studies, *in vitro* biochemical characterization of mutant and revertant paramyosins(e.g. the periodicity of paracrystals, formation rate of paracrystals), will be useful for a direct understanding the role of paramyosin in thick filament assembly.

None of the mutations sequenced affected regions outside the α-helical coiled-coil portion of the molecule. Paramyosin has a 30 amino acid N-terminal head and a 13 residue C-terminal tail. The head is probably phosphorylated, a modification in other systems known to affect assembly of coiled-coils of smooth muscle myosin(Bennett et al., 1988), Dictyostelium myosin(Pagh et al., 1984) and vimentin(Inagaki et al., 1987). The analysis of additional defective unc-15 mutants might uncover changes in these regions and help to elucidate their function. In reversion analysis one might also hope to find mutations in genes for interacting proteins which change the sequence, not just the quantity of protein. These extragenic suppressor mutations could be critical in dissecting the complex process of in vivo thick filament assembly.

6. Conclusion

In this work, an important fact was revealed, that is the defective function of altered paramyosin caused by the reversal of an electric-charge can be restored by the second mutations resulting in the charge changes of other residues. In the case of the molecules having α-helical coiled coil structure, such examples have been also shown in BicD protein of Drosophila. By superimposing the substituted amino acids of e73 and its suppressor alleles on the assembly model, it was postulated that the charge changes caused by these mutations may alter the affinity of paramyosin for itself. As described in the discussion, there seems to be some possibilities about how a single charge change on the helical structure can alter the assembly property in vivo, but it is still unclear. α-helical coiled-coil structure also exist in many other proteins of biological interest, such as mammalian transcription facters, receptors, and ion channel molecules, and thus α-helical coiled-coil interaction will be an important theme should be solved in the future.

This work showed charge changes of single amino acids can affect molecular assembly, and further affect the animal behavior. Such combined molecular and genetic approach should be useful in understanding not only thick filament assembly but also molecular assembly in other organelles.

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Acknowledgement

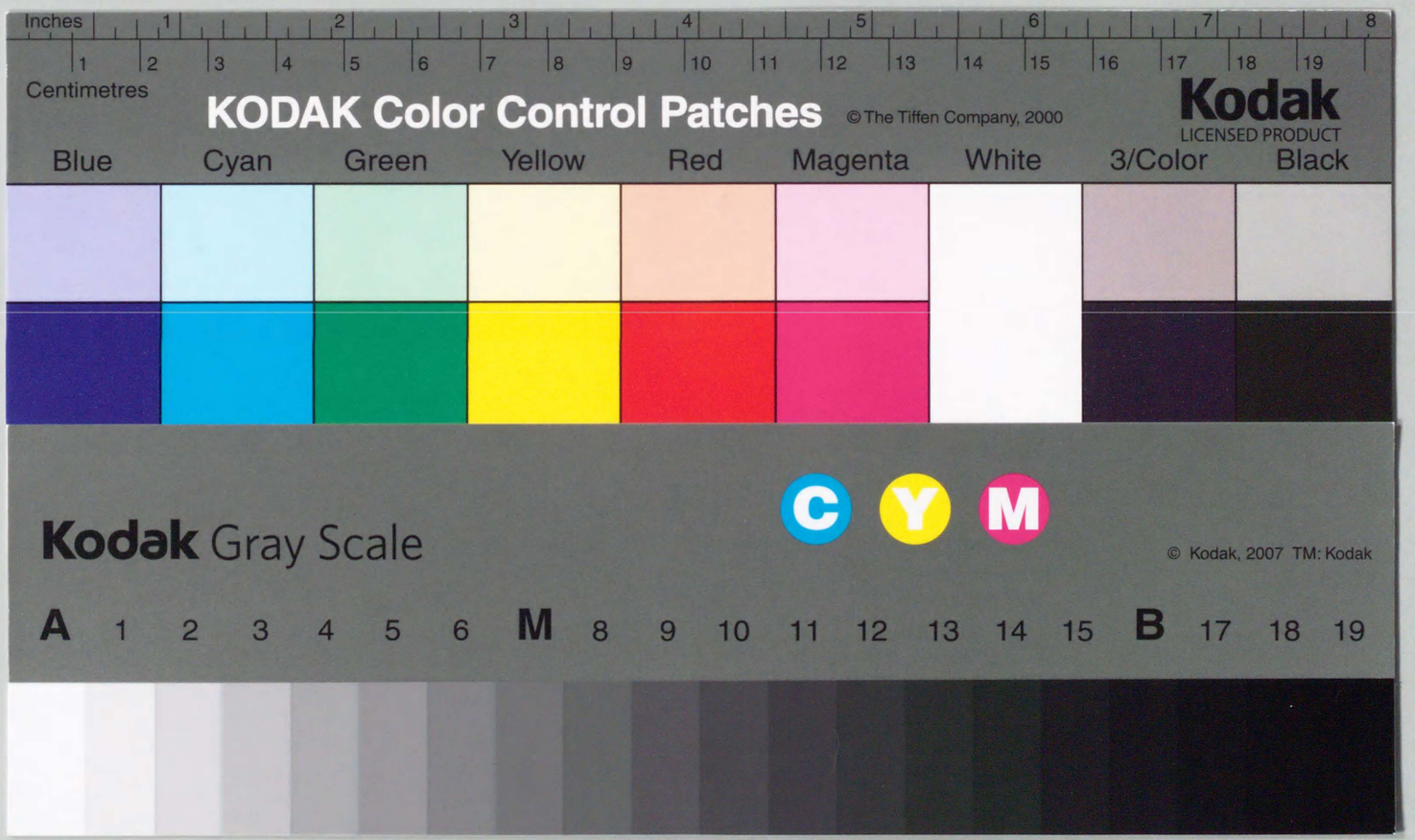
This work was mainly done at the Laboratory of Genetics and Cytology. I thank Dr. Enomoto and the members who work here. The radioisotope experiments of the present work were carried out in the Radio-Isotope Laboratory(Tsushima) of Okayama University.

I thank personally two people, my supervisor Dr. Kagawa for his generous guidance in research, and Dr. Waterston, who gave me acute suggestions for this work. I would also like to thank Dr. Sato for his encouragements. Drs. Rose and Riddle generously provided us unc-15 mutants. Other mutants and revertants were obtained from the Caenorhabditis Genetics Center which is supported by contract number N01-AG-9-2113 between the National Institutes of Health and the Curator of the University of Missouri. I thank the Laboratory of Animal Physiology for using a polarized light microscope, a part of polarized light micrographs were taken with it. The charge-mediated stagger model was largely developed by Dr. McLachlan. Drs. Kanazawa and Miki gave us good advice for direct sequencing, and Dr. Mori taught us a protochol to prepare genomic DNA. Antibodies were prepared by T. Lowe and S. Green, and synthetic oligonucleotide primers by S. Mukai(Ajinomoto Co. Ltd.). I thank J. Rothman and S. Green for teaching us immunofluorescence microscopy.

This work was supported by a Grant-in-Aid for Priority Area 02221101 to H.K. and by a Grant-in-Aid for Japanese Junior Scientists 02954213 to K.G.-A. from Ministry Education, Science

and Culture of Japan.

Finally, I would like to thank my husband, Hideki Ando, for his encouragements.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